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INVESTOR IN PEOPLE

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PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

REC'D 04 OCT 2000
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The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears an amendment, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

I also certify that by virtue of an assignment under the Patents Act 1977, the application is now proceeding in the name as substituted.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed Andrew Gersey

Dated 21 September 2000

GB9921337.3

By virtue of a direction given under Section 30 of the Patents Act 1977, the application is proceeding in the name of

THE DOW CHEMICAL COMPANY
2030 Dow Center
Midland
Michigan 48674
United States of America

Incorporated in USA - Delaware

[ADP No. 07348113001]

Patents Form 1/77

The Patent Office

10SEP99 E475697-2 D00032
P01/7700 0.00 - 9921337.3

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
09 SEP 1999
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Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference — JHL/JF/P13723GB

2. Patent application number
(The Patent Office will fill in this part) — 09 SEP 1999 — **9921337.3**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

AXIS GENETICS PLC.
Babraham
Cambridge
United Kingdom
CB2 4AZ

SECTION 30(1) 1977 ACT) APPLICATION FILED 02/02/00

Patents ADP number *(if you know it)* 8259167100/V

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention — "Modified plant viruses"

5. Name of your agent *(if you have one)* — ELKINGTON AND FIFE

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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LONDON
WC1A 2RA.

Patents ADP number *(if you know it)* ~~67004~~ 83001 V

FS1/77
15/2/2000
AP.

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of Filing *(day/month/year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of Filing *(day/month/year)*

Modified plant viruses

This invention relates to antigenic materials and more particularly to peptides rendered antigenic by being coupled to carrier substances. The invention is directed to the use of such coupled peptides to induce antibodies to certain tumour antigens.

Based on the success of vaccines for the treatment and prevention of certain infectious diseases, there have been attempts over the years to develop cancer vaccines. The first attempts at cancer vaccines were based on immunising the cancer patient with killed tumour cells together with adjuvant.

Further development of cancer vaccines has focused on directing the immune response to identified tumour antigens present on the surface of tumour cells. The advent of monoclonal antibodies enabled the identification of so-called "tumour-specific" antigens. In reality, tumour antigens are usually not tumour-specific but are over-expressed on tumour cells and rarely expressed, or under-expressed, on normal cells.

A tumour antigen that has been identified and studied is polymorphic epithelial cell mucin (PEM). PEM is the product of the MUC1 gene. PEM is over-expressed and aberrantly glycosylated in many carcinomas (breast, pancreas, ovary, lung, urinary bladder, prostate, and endometrium) thus resulting in cancer cells expressing antigenically distinct PEM molecules on their surface. Both humoral and cellular immune responses specific to PEM have been detected in cancer patients. These properties make PEM particularly well-suited to act as a target molecule for cancer immunotherapy. In addition, the over-expression of mucin on tumour cells suggests that the mucin may be important for the maintenance of the tumour. Such over-expression of mucin would alleviate potential problems of tumour cells escaping immunotherapy by down-regulating the MUC1 gene. The following patents and patent applications describe the purification of human mucin, the isolation of monoclonal antibodies thereto, the cloning of cDNAs coding for human mucin and the use of the above for diagnostic and therapeutic applications: US 4,963,484, US 5,053,489 and WO88/05054.

Zhang, S., *et al.* (Cancer. Res. 1996, 56, 3315-3319) teaches the preparation of and compares the immunogenicity of several MUC1 peptide vaccines. Vaccines comprising adjuvant and a MUC 1 peptide covalently linked to the protein carrier keyhole limpet

naked MUC1 DNA; and anti-MUC1 antibodies.

Of these, anti-MUC1 antibodies, synthetic carbohydrates, recombinant animal viruses, and peptides based on the tandem repeat have been developed sufficiently to reach evaluation in clinical trials. However, one significant problem with all of the above approaches is that a poor and/or inadequate immune response towards mucin-expressing tumour cells is observed.

The present invention alleviates the above-identified problem by providing a novel means for presenting a peptide of a tumour-associated mucin.

According to a first aspect of the invention, there is provided a chimaeric virus particle derived from a plant virus having a coat protein with a beta barrel structure and modified by insertion of an immunogenically active peptide of a tumour-associated mucin at an immunogenically effective site in the coat protein. The present invention provides, *inter alia*, the following advantages over the above-identified prior art antigen-presenting means. Conventional live animal virus vectors (as carriers) can be dispensed with; and the need for separate mucin peptide synthesis and the need for time-consuming chemical-coupling thereof to a conventional carrier, such as keyhole limpet haemocyanin (KLH), can be avoided.

We have found that surprisingly superior immunogenicity to a mucin antigen can be obtained by presenting a mucin peptide in a composite particle derived from certain plant viruses. The plant viruses especially useful for the purposes of the present invention are those having a coat protein with a beta barrel structure containing loops between beta sheets. Composite virus particles of this general type and the methods of constructing such particles are disclosed in our international applications WO92/18618 and WO96/02649, the contents of which are hereby incorporated for reference.

An advantage of the use of viruses which have a ß-barrel structure is that the loops between the individual strands of ß-sheet provide convenient sites for the insertion of mucin peptides. Modification of one or more loops is a preferred strategy for the expression of mucin peptides in accordance with the present invention. These viruses include all members of the following virus families: *Caulimoviridae, Bromoviridae, Comoviridae, Geminiviridae,*

Name	Acronym	Genus	Family
Southern bean mosaic virus	SBMV	*Sobemovirus*	not assigned
Sesbania mosaic virus	SMV	*Sobemovirus*	not assigned
tomato bushy stunt virus	TBSV	*Tombusvirus*	*Tombusviridae*
turnip crinkle virus	TCV	*Carmovirus*	*Tombusviridae*
cowpea chlorotic mottle virus	CCMV	*Bromovirus*	*Bromoviridae*
alfalfa mosaic virus	AMV	*Alfamovirus*	*Bromoviridae*
bean pod mottle virus	BPMV	*Comovirus*	*Comoviridae*
cowpea mosaic virus	CPMV	*Comovirus*	*Comoviridae*
red clover mottle virus	RCMV	*Comovirus*	*Comoviridae*
tobacco ringspot virus	TRSV	*Nepovirus*	*Comoviridae*
turnip yellow mosaic	TYMV	*Tymovirus*	not assigned
tobacco necrosis virus	TNV	*Necrovirus*	*Tombusviridae*
satellite tobacco necrosis virus		*Subgroup 2*	
satellite panicum mosaic virus		*Subgroup 2*	
satellite tobacco mosaic virus		*Subgroup 2*	

The similarity of the secondary structural elements and their spatial organisation is illustrated by Fig.1. Any of the loops which lie between the β-strands can be used for insertion of foreign epitopes, however the insertions are made such that the additions are exposed on either the internal or external surface of the virus and such that assembly of the coat protein subunits and the infectivity of the virus are not abolished. The choice of a particular loop can be made using knowledge of the structure of individual coat protein subunits and their interactions with each other, as indicated by the crystal structure, such that any insertions are unlikely to interfere with virus assembly. The choice of precise insertion site can be made, initially, by inspection of the crystal structure, followed by *in vivo* experimentation to identify the optimum site.

that the 3-D structures of BPMV and CPMV are very similar and are typical of the *Comoviridae* in general.

In the structures of CPMV and BPMV, each ß-barrel consists principally of 8 strands of antiparallel ß-sheet connected by loops of varying length. The connectivity and nomenclature of the strands is given in Figure 2 of WO 92/18618. The flat ß-sheets are named the B,C,D,E,F,G,H and I sheets, and the connecting loops are referred to as the ßB-ßC, ßD-ßE, ßF-ßG and ßH-ßI loops.

One difference between the *Comoviridae* and the animal *Picornaviridae* is that the protein subunits of *Comoviridae* lack the large insertions between the strands of the ß-barrels found in *Picornaviridae*. The four loops (ßB-ßC, ßD-ßE, ßF-ßG and ßH-ßI - see Figure 3 in WO 92/18618) between the ß-sheets are suitable for expression of tumour-associated mucin peptide sequences.

The ßB-ßC loop in the small capsid protein is particularly preferred as the insertion site. This loop has an engineered *Aat*II site and a unique *Nhe*1 site at position 2708 of the M RNA-specific sequence where mucin peptide sequences may be inserted (see Figure 4 of WO 92/18618). The insertion site immediately preceding Pro^{23} in the ßB-ßC loop of the small capsid protein is most preferred.

To demonstrate the present invention, the plant virus CPMV in particular has been primarily chosen.

Various sites in the CPMV coat protein have been identified as suitable for insertion of the foreign peptide. The co-ordinates given below refer to the linear amino acid sequence of the CPMV coat protein (S or L subunit).

Any insertion site which does not lie between the N-terminus of a subunit and a ß-strand, or between a ß-strand and the C-terminus, is considered to lie between two ß-strands. Such an insertion site may lie in a short loop at one of the axes of symmetry of the virus or in one of the much longer connecting strands which form the body of the protein subunits and which

The B domain of the L subunit comprises amino acids 183-374 of the linear amino-acid sequence.

ßB-ßC:

This site is in the equivalent location on the subunit to the standard S protein insertion site and is at the three-fold axis of the virus. The residues between the ß-strands are Pro 201 to Glu 209.

ßH-ßI:

Again this site is at the three-fold axis of the virus and the residues between the ß-strands are His 331-Asp 341.

ßC-αA (ßC-ßD):

This site lies between the ßC and ßD strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α-helix (termed αA) and the insertion site is a surface exposed portion which lies between the ßC strand and the αA helix. The surface exposed residues are Ala 223 to Ala 226.

ßG-αD (ßG-ßH):

This site lies between the ßG and ßH strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α-helix (termed αD) and the insertion site is a surface portion which lies between the αD helix and ßH strands which are surface exposed. The surface exposed residues are Pro 314 to Thr 317.

ßE-αB (ßE-ßF):

This site lies between the ßE and ßF strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α-helix (termed αB) and the insertion site is a surface portion which lies between the ßE strand and the αB helix. The surface exposed residues are Gly 269 to Phe 275.

L subunit, C Domain Insertion Sites

The C Domain of the L Subunit comprises amino-acids 1-182 of the linear amino-acid

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The Patent Office
Concept House
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Newport
South Wales
NP10 8QQ

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I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears an amendment, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

I also certify that by virtue of an assignment under the Patents Act 1977, the application is now proceeding in the name as substituted.

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Signed Andrew Gersey

Dated 21 September 2000

GB9921337.3

By virtue of a direction given under Section 30 of the Patents Act 1977, the application is proceeding in the name of

THE DOW CHEMICAL COMPANY
2030 Dow Center
Midland
Michigan 48674
United States of America

Incorporated in USA - Delaware

[ADP No. 07348113001]

Patents Form 1/77

The Patent Office

Patents Act 1977
(Rule 16)

THE PATENT OFFICE 09 SEP 1999 RECEIVED BY HAND

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P01/7700 0.00 - 9921337.3

Request for grant of a patent

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The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference — JHL/JF/P13723GB

2. Patent application number *(The Patent Office will fill in this part)* — 09 SEP 1999 — **9921337.3**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

AXIS GENETICS PLC.
Babraham
Cambridge
United Kingdom
CB2 4AZ

SECTION 32(1) 1977 ACT) APPLICATION FILED 02/02/00

Patents ADP number *(if you know it)* 05916710001

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention — "Modified plant viruses"

5. Name of your agent *(if you have one)* — ELKINGTON AND FIFE

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Carpmeals & Ransford
43, Bloomsbury Square
LONDON
WC1A 2RA.

Patents ADP number *(if you know it)* 67004 83001

FS1/77 15/2/2000 AP.

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of Filing *(day/month/year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of Filing *(day/month/year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer "Yes" if:*
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.

See note (d))

Yes

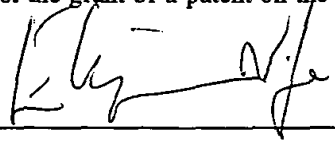
9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	0
Description	36
Claim(s)	3
Abstract	1
Drawing(s)	17 +17

10. If you are also filing any of the following, state how many against each item.

Priority documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	0
Request for preliminary examination and search *(Patents Form 9/77)*	0
Request for substantive examination *(Patents Form 10/77)*	0
Any other documents *(please specify)*	0

11. I/We request the grant of a patent on the basis of this application.

Signature  Date 09.09.99

12. Name and daytime telephone number of person to contact in the United Kingdom

Mr John H. Lewin
01732 458881

Modified plant viruses

This invention relates to antigenic materials and more particularly to peptides rendered antigenic by being coupled to carrier substances. The invention is directed to the use of such coupled peptides to induce antibodies to certain tumour antigens.

Based on the success of vaccines for the treatment and prevention of certain infectious diseases, there have been attempts over the years to develop cancer vaccines. The first attempts at cancer vaccines were based on immunising the cancer patient with killed tumour cells together with adjuvant.

Further development of cancer vaccines has focused on directing the immune response to identified tumour antigens present on the surface of tumour cells. The advent of monoclonal antibodies enabled the identification of so-called "tumour-specific" antigens. In reality, tumour antigens are usually not tumour-specific but are over-expressed on tumour cells and rarely expressed, or under-expressed, on normal cells.

A tumour antigen that has been identified and studied is polymorphic epithelial cell mucin (PEM). PEM is the product of the MUC1 gene. PEM is over-expressed and aberrantly glycosylated in many carcinomas (breast, pancreas, ovary, lung, urinary bladder, prostate, and endometrium) thus resulting in cancer cells expressing antigenically distinct PEM molecules on their surface. Both humoral and cellular immune responses specific to PEM have been detected in cancer patients. These properties make PEM particularly well-suited to act as a target molecule for cancer immunotherapy. In addition, the over-expression of mucin on tumour cells suggests that the mucin may be important for the maintenance of the tumour. Such over-expression of mucin would alleviate potential problems of tumour cells escaping immunotherapy by down-regulating the MUC1 gene. The following patents and patent applications describe the purification of human mucin, the isolation of monoclonal antibodies thereto, the cloning of cDNAs coding for human mucin and the use of the above for diagnostic and therapeutic applications: US 4,963,484, US 5,053,489 and WO88/05054.

Zhang, S., *et al.* (Cancer. Res. 1996, 56, 3315-3319) teaches the preparation of and compares the immunogenicity of several MUC1 peptide vaccines. Vaccines comprising adjuvant and a MUC 1 peptide covalently linked to the protein carrier keyhole limpet

haemocyanin achieved greatest immunogenicity. Zhang, S., *et al.* concluded that MUC 1 peptides of 30 or more amino acids are better immunogens than MUC 1 peptides of 20 amino acids.

The MUC1 gene product is a high molecular weight (Mr >200,000) transmembrane glycoprotein expressed on the apical surface of many simple epithelial cells. It has a relatively large extracellular domain varying from 1000 to 2200 amino acids and a cytoplasmic tail of 69 amino acids. The extracellular domain consists mainly of tandem repeats of the 20 amino acid sequence PDTRPAPGSTAPPAHGVTSA [for a review, see Apostolopoulos, V. and McKenzie, I.F.C. (1994) *Crit. Rev. Immunol.*, 14, pp.293-309]. Variability in the number of repeats accounts for the variability in the size of the extracellular domain. Each repeat contains five potential O-linked glycosylation sites and two or possibly three of these sites are believed to be utilised.

The aberrant glycosylation of PEM observed in a variety of cancers is due to alterations in the activity of glycosyl transferases with some transferases being inactive and the activity of others being increased. Abnormal glycosylation of PEM in cancer cells leads to three types of cancer-associated epitopes:
peptides, from the mucin core protein, that are exposed following under-glycosylation;
"new" carbohydrates resulting from deficient and aberrant glycosylation;
"new" glycopeptides resulting from deficient and aberrant glycosylation.

Many different approaches have been considered as potential cancer vaccines aimed at inducing a beneficial immune response directed towards PEM-expressing tumour cells.
These include:
cells expressing mucins;
mucin purified from cancer cells;
mucin core protein produced as a recombinant protein;
different mucin glycoproteins produced by transfected cells;
peptide or glycopeptide based on the tandem repeat;
recombinant animal viruses expressing portions of the MUC1 cDNA gene product;
synthetic carbohydrates based on short aberrant chains present on mucins on cancer cells;

naked MUC1 DNA; and anti-MUC1 antibodies.

Of these, anti-MUC1 antibodies, synthetic carbohydrates, recombinant animal viruses, and peptides based on the tandem repeat have been developed sufficiently to reach evaluation in clinical trials. However, one significant problem with all of the above approaches is that a poor and/or inadequate immune response towards mucin-expressing tumour cells is observed.

The present invention alleviates the above-identified problem by providing a novel means for presenting a peptide of a tumour-associated mucin.

According to a first aspect of the invention, there is provided a chimaeric virus particle derived from a plant virus having a coat protein with a beta barrel structure and modified by insertion of an immunogenically active peptide of a tumour-associated mucin at an immunogenically effective site in the coat protein. The present invention provides, *inter alia*, the following advantages over the above-identified prior art antigen-presenting means. Conventional live animal virus vectors (as carriers) can be dispensed with; and the need for separate mucin peptide synthesis and the need for time-consuming chemical-coupling thereof to a conventional carrier, such as keyhole limpet haemocyanin (KLH), can be avoided.

We have found that surprisingly superior immunogenicity to a mucin antigen can be obtained by presenting a mucin peptide in a composite particle derived from certain plant viruses. The plant viruses especially useful for the purposes of the present invention are those having a coat protein with a beta barrel structure containing loops between beta sheets. Composite virus particles of this general type and the methods of constructing such particles are disclosed in our international applications WO92/18618 and WO96/02649, the contents of which are hereby incorporated for reference.

An advantage of the use of viruses which have a ß-barrel structure is that the loops between the individual strands of ß-sheet provide convenient sites for the insertion of mucin peptides. Modification of one or more loops is a preferred strategy for the expression of mucin peptides in accordance with the present invention. These viruses include all members of the following virus families: *Caulimoviridae, Bromoviridae, Comoviridae, Geminiviridae,*

Reoviridae, Partitiviridae, Sequiviridae, Tombusviridae, and the following virus genera: Luteovirus, Marafivirus, Sobemovirus, Tymovirus, Enamovirus and Idaeovirus. Of the *Tombusviridae* family, the following genera are mentioned in particular: Dianthovirus, Machlomovirus and Necrovirus. An advantage of the *Comoviridae* and *Sequiviridae* is that their capsid contains sixty copies each of 3 different ß-barrels which can be individually manipulated. All other virus families and genera listed above have similar 3-dimensional structures but with a single type of ß-barrel. Viruses selected from the family *Comoviridae* (e.g. cowpea mosaic virus (CPMV), and bean pod mottle virus) are particularly preferred. CPMV is the most preferred virus.

The invention can be applied to any plant virus having a coat protein with a ß-barrel structure. In a preferred embodiment the three dimensional structure of a plant virus is examined in order to identify portions of a coat protein which are particularly exposed on the virus surface and which are therefore potentially optimum sites for insertion. In a further embodiment the amino acid sequence of the exposed portions of a coat protein is examined for amino acids which break α-helical structures because these are potentially optimum sites for insertion. Examples of suitable amino acids are proline and hydroxyproline, both of which whenever they occur in a polypeptide chain interrupt the α-helix and create a rigid kink or bend in the structure.

All plant viruses possessing icosahedral symmetry whose structures have been solved conform to the eight stranded β-barrel fold as exemplified by cowpea mosaic virus, and it is likely that this represents a common structure in all icosahedral viruses. All such viruses are suitable for use in this invention for the presentation of foreign peptide sequences in the loops between the ß-strands.

To date, viruses from nine plant virus genera and three subgroup 2 ssRNA satellite viruses have had their tertiary and quaternary structures solved at high resolution. These are:

Name	Acronym	Genus	Family
Southern bean mosaic virus	SBMV	*Sobemovirus*	not assigned
Sesbania mosaic virus	SMV	*Sobemovirus*	not assigned
tomato bushy stunt virus	TBSV	*Tombusvirus*	*Tombusviridae*
turnip crinkle virus	TCV	*Carmovirus*	*Tombusviridae*
cowpea chlorotic mottle virus	CCMV	*Bromovirus*	*Bromoviridae*
alfalfa mosaic virus	AMV	*Alfamovirus*	*Bromoviridae*
bean pod mottle virus	BPMV	*Comovirus*	*Comoviridae*
cowpea mosaic virus	CPMV	*Comovirus*	*Comoviridae*
red clover mottle virus	RCMV	*Comovirus*	*Comoviridae*
tobacco ringspot virus	TRSV	*Nepovirus*	*Comoviridae*
turnip yellow mosaic	TYMV	*Tymovirus*	not assigned
tobacco necrosis virus	TNV	*Necrovirus*	*Tombusviridae*
satellite tobacco necrosis virus		*Subgroup 2*	
satellite panicum mosaic virus		*Subgroup 2*	
satellite tobacco mosaic virus		*Subgroup 2*	

The similarity of the secondary structural elements and their spatial organisation is illustrated by Fig.1. Any of the loops which lie between the β-strands can be used for insertion of foreign epitopes, however the insertions are made such that the additions are exposed on either the internal or external surface of the virus and such that assembly of the coat protein subunits and the infectivity of the virus are not abolished. The choice of a particular loop can be made using knowledge of the structure of individual coat protein subunits and their interactions with each other, as indicated by the crystal structure, such that any insertions are unlikely to interfere with virus assembly. The choice of precise insertion site can be made, initially, by inspection of the crystal structure, followed by *in vivo* experimentation to identify the optimum site.

The present invention is also applicable to those β-barrel containing plant viruses whose crystal structures have not yet been determined. Where significant sequence homology within the coat protein genes exists between one virus whose crystal structure is unknown and a second virus whose crystal structure has been determined, alignment of the primary structures will allow the locations of the loops between the β-strands to be inferred [see Dolja, V.V. and Koonin, E.V. (1991) J. Gen. Virol., 72, pp 1481-1486]. In addition, where a virus has only minimal coat protein sequence homology to those viruses whose crystal structure has been determined, primary structural alignments may be used in conjunction with appropriate secondary and tertiary structural prediction algorithms to allow determination of the location of potential insertion sites.

CPMV comprises two subunits, the small (S) and the large (L) coat proteins, of which there are 60 copies of each per virus particle. Mucin peptide sequences may be expressed on either the L or S proteins or on both coat proteins on the same virion. Thus, up to 120 copies of the mucin peptide sequence may be expressed on a single virus particle.

A 3.5Å electron density map of CPMV (see Figure 1 in WO92/18618) shows the clear structural relationship between the capsids of CPMV and the T=3 plant viruses, for example the bromoviruses, in particular cowpea chlorotic mottle virus (CCMV) and the sobemoviruses, in particular southern bean mosaic virus (SBMV). The capsids of these latter viruses are composed of 180 identical coat protein subunits, each consisting of a single ß-barrel domain. These domains can occupy three different positions, namely A, B and C, within the virions (see Figure 1 in WO 92/18618). The two coat proteins of CPMV were shown to consist of three distinct ß-barrel domains, two being derived from the large capsid protein and one from the small capsid protein. Thus, in common with the T=3 viruses, each CPMV particle is made up of 180 ß-barrel structures. The single domain from the small subunit occupies a position analogous to that of the A type subunits of CCMV and SBMV and other viruses, whereas the N- and C-terminal domains of the large capsid protein occupy the positions of the C and B type subunits respectively (see Figure 1 in WO 92/18618).

X-ray diffraction analysis of crystals of CPMV and bean pod mottle virus (BPMV) shows

that the 3-D structures of BPMV and CPMV are very similar and are typical of the *Comoviridae* in general.

In the structures of CPMV and BPMV, each ß-barrel consists principally of 8 strands of antiparallel ß-sheet connected by loops of varying length. The connectivity and nomenclature of the strands is given in Figure 2 of WO 92/18618. The flat ß-sheets are named the B,C,D,E,F,G,H and I sheets, and the connecting loops are referred to as the ßB-ßC, ßD-ßE, ßF-ßG and ßH-ßI loops.

One difference between the *Comoviridae* and the animal *Picornaviridae* is that the protein subunits of *Comoviridae* lack the large insertions between the strands of the ß-barrels found in *Picornaviridae*. The four loops (ßB-ßC, ßD-ßE, ßF-ßG and ßH-ßI - see Figure 3 in WO 92/18618) between the ß-sheets are suitable for expression of tumour-associated mucin peptide sequences.

The ßB-ßC loop in the small capsid protein is particularly preferred as the insertion site. This loop has an engineered *Aat*II site and a unique *Nhe*1 site at position 2708 of the M RNA-specific sequence where mucin peptide sequences may be inserted (see Figure 4 of WO 92/18618). The insertion site immediately preceding Pro^{23} in the ßB-ßC loop of the small capsid protein is most preferred.

To demonstrate the present invention, the plant virus CPMV in particular has been primarily chosen.

Various sites in the CPMV coat protein have been identified as suitable for insertion of the foreign peptide. The co-ordinates given below refer to the linear amino acid sequence of the CPMV coat protein (S or L subunit).

Any insertion site which does not lie between the N-terminus of a subunit and a ß-strand, or between a ß-strand and the C-terminus, is considered to lie between two ß-strands. Such an insertion site may lie in a short loop at one of the axes of symmetry of the virus or in one of the much longer connecting strands which form the body of the protein subunits and which

may contain additional secondary structure and form loops on the surface of the virus. In particular, there are α-helices present in some of the connecting strands which form the body of the protein subunits, and the co-ordinates given for some of the insertion sites may indicate that an α-helix is present between the insertion site and the preceding or proceeding ß-strand. For example, the S protein C' and C" ß-strands represent a secondary structure formed in the loop between the ßC and ßD strands.

(i) External Surface Sites

S Subunit (A Domain) Insertion Sites

ßB-ßC:

The residue between the ß-strands are Thr 19 to Val 2, and the preferred insertion site is between amino-acids 22 and 23. Insertion sites either side of this are also suitable, notably between residues 21 and 22.

ßC'-ßC":

The residues between the ß-strands are Val 42 to Asn 46.

ßH-ßI:

This site is at the tip of the five-fold axis and the residues between the ß-strands are Thr 152 to Gln 158.

ßD-ßE:

Again, this site is at the tip of the five-fold axis and the residues between the ß-strands are Ala 80 to Gln 90.

ßE-ßF:

This site is not at the tip of the five-fold axis, but lies 'behind' and to one side of the ß-strands. The residues between the ß-strands are Arg 96 to Ala 106. Residues 98 to 102 are preferred.

L Subunit, B Domain Insertion Sites

The B domain of the L subunit comprises amino acids 183-374 of the linear amino-acid sequence.

ßB-ßC:

This site is in the equivalent location on the subunit to the standard S protein insertion site and is at the three-fold axis of the virus. The residues between the ß-strands are Pro 201 to Glu 209.

ßH-ßI:

Again this site is at the three-fold axis of the virus and the residues between the ß-strands are His 331-Asp 341.

ßC-αA (ßC-ßD):

This site lies between the ßC and ßD strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α-helix (termed αA) and the insertion site is a surface exposed portion which lies between the ßC strand and the αA helix. The surface exposed residues are Ala 223 to Ala 226.

ßG-αD (ßG-ßH):

This site lies between the ßG and ßH strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α-helix (termed αD) and the insertion site is a surface portion which lies between the αD helix and ßH strands which are surface exposed. The surface exposed residues are Pro 314 to Thr 317.

ßE-αB (ßE-ßF):

This site lies between the ßE and ßF strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α-helix (termed αB) and the insertion site is a surface portion which lies between the ßE strand and the αB helix. The surface exposed residues are Gly 269 to Phe 275.

L subunit, C Domain Insertion Sites

The C Domain of the L Subunit comprises amino-acids 1-182 of the linear amino-acid

sequence.

ßE- αB (ßE-ßF):

This site lies between the ßE and ßF strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α-helix (termed αB) and the insertion site is a surface exposed portion which lies between the ßE strand and the αB helix. The surface exposed residues are Gly 95 to Thr 102.

αD-ßH (ßG-ßH):

This site lies between the ßG and ßH strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α-helix (termed αD) and the insertion site is a surface portion which lies between the αD helix and the ßH strands. The surface exposed residues are Ser 142 and Arg 145.

ßC-αA (ßC-ßD):

This site lies between the ßC and ßD strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α-helix (termed αA) and the insertion site is a surface exposed portion which lies between the ßC strand and the αA helix. The surface exposed residues, not part of any secondary structural element, are Gly 53 to Phe 56.

ßB-ßC:

This site is an equivalent location on this domain to the S protein ßB-ßC (identified above) insertion site and is at the three-fold axis of the virus. The residues between the ß-strands are Ser 33 to Leu 42.

(ii) **Internal Surface Sites**

S Subunit (A Domain) Insertion Sites

ßG-ßH:

This protein chain between ß-strands points in towards the interior of the virus and forms a `double loop´. One insertion site comprises residues Pro 128 to Ser 130.

L Subunit B Domain Insertion Sites

ßF-ßG:

This loop is at the three-fold axis symmetry of the virus, and is the bottom loop of the four. The residues in the loop are Gln 287 to Glu 293.

C Domain ßl-B Domain ßB:

This is the junction between the B and C domains of the L subunit. This linker sequence comprises residues Asn 374 to Asp 186. The insertion site is around Ala 185, which is assigned to the B domain.

L Subunit, C Domain Insertion Sites

ßG-αD (ßG-ßH):

This site lies between the ßG and ßH strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α-helix (termed αD) and the insertion site is an internal projecting loop which lies between the ßG strands and the αD helix. The residues in this loop are Asn 130 to Ser 135.

The RNA or DNA encoding the mucin peptide may be inserted into the plant virus genome in a variety of configurations. For example, it may be inserted as an addition to the existing nucleic acid or as a substitution for part of the existing sequence, the choice being determined largely by the structure of the capsid protein and the ease with which additions or replacements can be made without interference with the capacity of the genetically-modified virus to assemble. Determination of the permissible and most appropriate size of addition or deletion for the purposes of this invention may be achieved in each particular case by experiment in the light of the present disclosure. The use of addition inserts appears to offer more flexibility than replacement inserts in some instances.

The mucin peptides which may be incorporated into plant viruses according to this invention may be of highly diverse types and are subject only to the limitation that the nature and size of the peptide and the site at which it is placed on or in the virus particle do not interfere

which the capacity of the modified virus to assemble when cultured *in vitro* or *in vivo*. The peptide preferably contains 5 or more amino acids.

The following are preferred mucin peptides sequences for forming CVPs in accordance with the present invention. Optionally repeating sequences of the 20 amino acid sequence "PDTRPAPGSTAPPAHGVTSA" (SEQ. ID No.1) are preferred. An optionally repeating partial sequence of the above 20 amino acid sequence is particularly preferred. In this respect the peptide sequences "PDTRP" (SEQ. ID No.2) and "APDTR" (SEQ. ID No.3) are particularly preferred, as are the mimetic peptide sequences: "DAHWESWL" (SEQ. ID No.4) and "DLHWASWV" (SEQ. ID No.5).

The mucin peptide may be of the general formula "$(aa)_x$PDTRP$(aa)_y$", or "$(aa)_x$APDTR$(aa)_y$" where aa is an amino acid residue, the same or different in each position, x is an integer from 0 to 1000, and y is an integer from 0 to 1000. Preferably x is an integer from 0 to 500, more preferably 0 to 100, most preferably 0 to 10 and y is an integer from 0 to 500, more preferably 0 to 100, most preferably 0 to 10.

It is most preferred that the sequence "PDTRP" or "APDTR" is located towards the middle of the mucin peptide sequence of interest (as for example in the MUC1(16) defined hereinafter). Alternatively, the peptide sequence "PDTRP" or "APDTR" may be located towards the beginning of the mucin peptide sequence of interest (as for example in the MUC1(23) defined hereinafter). The above sequences/partial sequences are based on the 20 amino acid tandem repeat sequence of the extracellular of PEM.

According to a second aspect of the invention, there is provided a method of producing a chimaeric virus particle which comprises introducing a nucleotide sequence coding for a tumour-associated mucin peptide to modify the plant viral nucleic acid which codes for the coat protein, infecting plants, plant tissue, plant cells, or protoplasts with the modified viral nucleic acid, and harvesting chimeric virus particles. The introduced nucleotide sequence is preferably inserted in that part of the plant viral nucleic acid which codes for an exposed region of the coat protein.

This procedure is best carried out by direct manipulation of the DNA of the virus in the case of DNA viruses or by manipulation of the cDNA corresponding to the RNA of an RNA virus. In the case of an RNA virus, the modified cDNA or an RNA transcript thereof is prepared for inoculation of plant cells or preferably whole plants so as to achieve a multiplication stage prior to the harvesting of assembled particles of the modified virus. In the case of a DNA virus, the DNA itself is introduced into the plant. In this way, the mucin peptide is initially expressed as part of the capsid protein and is thereby produced as part of the whole virus particle.

In order to produce modified virus on a commercial scale, it is not necessary to prepare infective inoculant (DNA or RNA transcript) for each batch of virus production. Instead, an initial inoculant may be used to infect plants and the resulting modified virus may be passaged in the plants to produce whole virus or viral RNA as inoculant for subsequent batches.

Preferably the method is applied to an RNA plant virus, in which case the method comprises introducing a DNA coding for the tumour-associated mucin peptide into a cDNA corresponding to the RNA of the plant virus which codes for an exposed portion of its coat protein, inoculating plants, plant tissue, plant cells, or protoplasts with the thus modified cDNA or an RNA transcript thereof, if necessary together with any other DNA or RNA required for multiplication and assembly of whole virus particles in the plant material, and harvesting chimaeric virus particles (CVPs). More preferably, the modified cDNA is produced by introducing the DNA encoding the mucin peptide into a DNA fragment excised from the plant viral cDNA, and reinserting the modified excised fragment so as to constitute the plant viral cDNA in modified form.

According to a third aspect of the invention, there is provided a vaccine comprising CVPs, as hereinbefore described, as an immunogenic component thereof. The vaccine may further comprise adjuvant, for example Freund's complete adjuvant (FCA), QuilA, QS-21, ISCOM matrix, algammulin, or combinations thereof. QS-21 is preferred. Alternatively, adjuvant may be omitted from the vaccine. It is particularly surprising that the chimaeric virus particles of the present invention are strongly immunogenic in the absence of an adjuvant (see

Example 8 and Fig 4).

Vaccines according to the present invention are particularly suited for nasal administration. For comparison purposes, a chimaeric plant virus particle comprising alfalfa mosaic virus coat protein and a rabies virus peptide is described in Modelska *et al* [Proc. Natl. Acad. Sci. USA 1998, 95, pp 2481-2485]. The chimeric plant virus coat protein assembled *in planta* into virus-like particles which (in contrast to the CVPs of the present invention) were non-infectious to plants. Three doses of 50 μg of the virus-like particles were required to obtain an immune response when administered to mice by injection in the absence of an adjuvant. In contrast, a strong immune response was achieved in accordance with the present invention by either subcutaneous or nasal administration to mice of two 100 μg doses of CPMV-MUC1(16) in the absence of an adjuvant (see Example 7 and Figure 4; and Example 8 and Figure 7).

The invention is illustrated by the accompanying drawings which are first described briefly, and the subsequent Examples which give further details. No limitation of the present invention to the Examples is intended.

Fig. 1. is a simple line drawing of all the solved β-barrel containing virus structures showing the secondary structural elements which make up the coat protein domains.

Fig 2. Subcutaneous immunisation with CPMV-MUC1(16) and CPMV-MUC1(23) in FCA induces MUC1- and CPMV-specific serum IgG.

C57BL/6 mice (8 per group) were immunised subcutaneously with 100 μg of (A) CPMV-MUC1(16), (B) CPMV-MUC1(23) or (C) wild type CPMV in Freund's complete adjuvant (FCA) and boosted on days 14 and 28 with 25 μg in incomplete Freund's adjuvant (IFA). Sera were collected on day 42 and tested for MUC1 60mer-specific and CPMV-specific IgG1/IgG2a (mixture of conjugates) by ELISA. Titres are expressed as end-point titres obtained with sera from individual mice. Results are of a single experiment representative of a further 2 experiments.

Fig 3. Sera from CPMV-MUC1(16)- and CPMV-MUC1(23)-immunised mice stain MUC1-expressing tumour cell lines by flow cytometry.

(A) C57BL/6 mice (8 per group) were immunised subcutaneously with 100 μg of CPMV-MUC1(16), CPMV-MUC1(23) or wild type CPMV (8 per group) in FCA and boosted on days 14 and 28 with 25 μg doses in IFA. Sera were collected on day 42, pooled, diluted 1:4 and used to stain MUC1-transfected mouse E4 cells and MUC1-expressing human T47D cells. A 1:4 dilution of mouse anti-human MUC1 mAb HMFG2 was used as a control. (B) C57BL/6 mice were immunised subcutaneously with 100 or 10 μg of CPMV-MUC1 or with 10 μg MUC1 conjugated to KLH in QS-21 and boosted with the same on day 21. Sera were collected on day 35, pooled, diluted 1:4 and used to stain mouse 410.4, E4 and T47D cells. Results are expressed as mean fluorescence intensity (MFI).

Fig. 4. CPMV is a more effective carrier of the MUC1 peptide than KLH even in the absence of adjuvant.

C57BL/6 mice (6 per group) were immunised subcutaneously with 100 μg of CPMV-MUC1(16) with and without QS-21, with 10 μg CPMV-MUC1(16) in QS-21 or with 10 μg of the MUC1 peptide conjugated to KLH in QS-21 and boosted with same on day 21. Sera were collected on day 37 and assayed for MUC1 60mer-specific IgG1/IgG2a by ELISA. Results are expressed as mean end-point titres (standard deviations shown) obtained with sera from individual mice.

Fig. 5. CPMV-MUC1(16) is a more effective carrier of the MUC1 peptide than KLH in human MUC1-transgenic mice.

C57BL/6 mice (5 per group) were immunised subcutaneously on day 0 with 100 μg of CPMV-MUC1(16) or wild type CPMV in QuilA and boosted with the same on day 14. C57BLx*Sac*II human MUC-1 transgenic mice were immunised in a similar manner using CPMV-MUC1(16) (6 mice), wild type CPMV (5 mice) and MUC1-KLH (3 mice), except that 25 μg doses of the MUC1 peptide conjugated to KLH were used for immunisation. Sera were collected on day 21 and analysed for anti-MUC1 antibodies by ELISA. Sera from mice of the same group were pooled except for the transgenic mice immunised with CPMV-MUC1(16), which were analysed as individual samples.

Fig. 6. The relative efficacy of adjuvants to augment MUC1-specific titres elicited by CPMV-MUC1(16).

C57BL/6 (5 per group) mice were immunised subcutaneously with 50 μg of CPMV-MUC1(16) and boosted on days 14 and 28 with 25 μg of CPMV-MUC1(16) in either FCA/FIA, QuilA, QS-21, ISCOM matrix or algammulin. Sera were collected on days 28, 42 and 150 (or 90 for algamulin) and assayed for MUC1 60mer-specific IgG1/IgG2a by ELISA. Titres are expressed as the mean end-point titre obtained with the sera from individual mice within each group. Standard deviations are shown for each value.

Fig. 7. Intranasal immunisation with CPMV-MUC1(16) induces MUC1(16)-specific IgG in serum.

C57BL/6 mice (5 per group) were immunised intranasally on days 0, 7, 14 and 21 with 100 μg of CPMV-MUC1(16) or wild type CPMV in the presence or absence of cholera toxin (CT). Sera and intesinal lavages were collected on day 42 and assayed for MUC1 peptide- and CPMV-specific IgG_1/IgG_{2a} (sera) and IgA (lavage) by ELISA. Titres for each mouse are expressed as end-point titres obtained with sera/lavage from individual mice.

Fig. 8 shows the nucleotide and protein sequences of SBMV surrounding a potential insertion site.

FIG. 9 shows a comparison of ßH-ßI loop of three sobemoviruses. Conserved residues are highlighted in bold and the locations of the loops and ß-strands are indicated.

Fig. 10 shows the nucleotide and protein sequences of LTSV surrounding a potential insertion site.

Fig. 11 illustrates alignment of the coat protein sequences of RCNMV and TBSV using a Lipman-Pearson alignment algorithm.

Fig. 12 illustrates a Chou-Fasman ß-region prediction plot of RCNMV residues 214-257 using an algorithm based upon the structures found in 64 proteins.

Fig. 13 illustrates application of the EMBL PHDsec algorithm program to the same RCNMV sequence as shown in Fig 12.

Fig. 14 shows the nucleotide and protein sequences of RCNMV surrounding a potential insertion site.

Examples

In the following studies (Examples 1-8) we have expressed in the ßB-ßC loop of the S protein of CPMV, either a 16-mer or a 23-mer peptide sequence derived from the MUC1 gene product tandem repeat. The corresponding chimaeric virus particles (CVPs) are hereinafter defined as CPMV-MUC1(16) and CPMV-MUC1(23), respectively. Similarly, the gene product of MUC1 is hereinafter described as MUC1 peptide (or simply MUC1). The CVPs were used to immunise mice and the MUC1-specific immune response was examined. The CVPs were shown to be highly efficient carriers of the MUC1 peptide sequences, eliciting high titres of anti-MUC1 antibodies that strongly recognised MUC1-bearing tumour cells and fresh breast tissue. Mucin-based chimeric plant viruses therefore have significant potential as cancer vaccines against mucin-expressing epithelial cancers.

Experimental Animals

Female C57BL/6 mice and C57BL6x*Sac*II MUC1-transgenic mice (both H-2^b), aged 6-8 weeks, were used in these studies.

Monoclonal antibodies (mAbs) and cell lines

HMFG2 is a MUC1-specific mouse IgG1 mAb (see reference 2). E4 cells are derived from the murine (BALB/c) mammary epithelial cancer cell line 410.4 transfected with the MUC1 gene. T47D is a human breast carcinoma cell line, obtained from ATCC (American Tissue Culture Collection; MD).

Peptides

A MUC1 60-mer peptide representing 3 tandem repeats was synthesised using an automated peptide synthesizer and used for ELISA. A MUC1 16-mer peptide, corresponding to the

sequence expressed on CPMV-MUC1 was synthesised and coupled to KLH (by the EDC method using a kit purchased from Pierce (Europe); coupling efficiency approximately 15-25 peptide copies per KLH molecule) by Genosys Biotechnologies (Europe), Cambridge, UK.

CPMV-MUC1 Construction

The genome of CPMV consists of two molecules of single stranded plus sense RNA, (RNA1 and RNA2) which were cloned on separate plasmids as full length cDNAs (pCP1 and pCP2 respectively - as described in reference 3).

(i) Construction of Vector pCP2-0.51

This vector is derived from pCP2 (see Example 6 of WO 96/02649) and was constructed as described in reference 4. The *Aat*II *s*ite present in the pUC vector sequences of pCP2 was removed by linearisation of the plasmid with this enzyme, followed by treatment with the Klenow fragment of *E. coli* DNA polymerase I to eliminate the 3' overhangs, followed by religation to form pCP2-*Aat*II. pCP2-*Aat*II was then digested with *Bam*HI and *Eco*RI and the 2kb fragment released was replaced by the equivalent fragment derived from pMT7-HRVII (see reference 5). The resultant plasmid, pCP2-0.51, therefore contained the mutated S coat protein gene containing an engineered *Aat*II restriction site together with additional sequences coding for a fragment of the coat protein of human rhinovirus (HRV) inserted between CPMV RNA2 nucleotides 2725 and 2726.

(ii) Construction of Vector pCP7

Vector pCP2-0.51 was digested with *Sac*II and *Eco*RI to release a DNA fragment carrying the CaMV 35S promoter fused to the full-length cDNA clone of CPMV RNA2. This fragment was ligated into similarly digested pBSII SK^+ (Stratagene) to make vector pCP7-HRVII. The HRV sequence together with CPMV flanking sequences were then excised by digestion with *Nhe*I and *Aat*II and replaced by a pair of chemically synthesised overlapping oligonucleotides carrying the CPMV flanking sequences only (see reference 6), thus restoring the normal CPMV sequence between these two restriction sites. All the cloning junctions described were verified by sequence analysis and the resultant vector was called pCP7. Subsequently the complete sequence of the CaMV35S-CPMV RNA2 cassette was determined between the *Sac*II and *Eco*RI restriction sites.

Construction of CPMV-MUC1(16) and CPMV-MUC1(23) CVPs

pCP7 was digested with *Nhe*I and *Aat*II and the excised fragment replaced by oligonucleotides coding for the excised wild-type CPMV sequences plus either a 16-mer MUC1 peptide "GVTSAPDTRPAPGSTA" (SEQ.ID No. 6) or a 23-mer MUC1 peptide "PDTRPAPGSTAPPAHGVTSAPDT" (SEQ. ID No. 7) forming plasmids pCP7-MUC1(16) and pCP7-MUC1(23), respectively.

Infection of Cowpea plants (*Vigna unguiculata* cv. Blackeye), characterisation and purification of recombinant virus particles.

Cowpea plants were inoculated with linearised pCP1 and pCP7-MUC1 as described in reference 3. DNA maxi-preps were made of each plasmid using the Qiagen purification system. 50 μg of pCP1 were digested with *Mlu*I and 50 μg pCP7-MUC1 were digested with *Eco*RI. The plasmids were purified by phenol/chloroform extraction and ethanol precipitation and each resuspended in 125 μl 10 mM sodium phosphate buffer pH 7.0. The plasmids were mixed together and 50 μl of the mixture were inoculated onto one primary leaf of each of five 10 day old cowpea plants by manual abrasion in the presence of carborundum powder.

All five inoculated plants developed chlorotic lesions in the inoculated leaves and a chlorotic mosaic on the secondary leaves. The recombinant virus spread throughout the growing plant in a manner similar to that of a natural infection with wild type CPMV. After 2-3 weeks, small samples (~ 10mg) of tissue were taken from the youngest leaves of each plant, which were showing good symptoms of viral infection. Each sample was ground in 400 μl 0.1 M sodium phosphate buffer, the extract was emulsified with an equal volume of a 1:1 mixture of chloroform/n-butanol, clarified by centrifugation and the aqueous phase was retained. 100 μl of 1 M NaCl/20% PEG 8000 were added, followed by incubation on ice for 20 minutes. The precipitate was collected by centrifugation for 5 min and resuspended in 20 μl sterile deionised water. 2 μl of each particle `mini-prep´ were then analysed by RT-PCR and agarose gel electrophoresis, and the products of the PCR reaction were sequenced. In all 5 plants, a single band of the expected size was seen in the RT-PCR reaction and the sequence data showed that the sequence of the inserted RNA was correct in each case.

The leaf material of all the plants was then harvested and the recombinant virus purified according to standard techniques (as described in Example 2 of WO 96/02649) and the final product sterilised by filtration through a 0.2 μm membrane. 5 μg of the purified virus were analysed by SDS-PAGE followed by staining with coomassie brilliant blue. A band corresponding to the virus L subunit was seen together with a band characteristic of the S coat protein containing an inserted peptide. The purified particles were also analysed via RT-PCR, and the PCR products further characterised by sequencing. The sequence of the inserted RNA was found to be correct.

The characterised particles from the first round of infection were designated as master stocks and used to initiate a second round of infection in a further five plants in order to generate a working stock (as described in reference 6). After 2-3 weeks the plant leaves were harvested and the recombinant virus purified and characterised as before - with the same results. This demonstrated that the inserted peptide sequence was stable over at least two passages.

Each CVP expressed 60 copies of the appropriate MUC1 peptide sequence. 1 µg CVP contains approximately 17 ng of the appropriate peptide sequence.

ELISA for detection of MUC1-specific antibody

ELISAs for the detection of MUC1-specific and CPMV-specific antibody in sera was performed as described in references 5 and 6. For the detection of anti-MUC1 antibody, the wells of 96-well ELISA plates (Dynatech Immulon-4) were coated with 0.1 μg/well of streptavidin (Sigma) for 1 hr at 37°C followed by 0.1 μg/well of biotinylated MUC1 60-mer peptide for 1 h at 37°C. For the detection of anti-CPMV antibody, the wells were coated directly with 0.1 μg/well of CPMV for 1 hr at 37°C. A series of doubling dilutions of serum were incubated on the antigen-coated plates for 1 and 4 h respectively at 37°C. Bound antibody was detected with alkaline phosphatase conjugated goat anti-mouse IgG1 and IgG2a (a mix of the two conjugates) or with IgA (Southern Biotechnologies Inc, USA), with p-nitrophenyl phosphate (PNPP) (Sigma) as the substrate. The products were quantified with an automated ELISA reader at 405 nm. For CPMV-specific titres, the results are expressed as an end-point titre, calculated as the inverse of the dilution that gave a mean OD_{405nm} higher

than the OD_{405nm} obtained with a 1:50 dilution of pooled serum from unimmunised mice. For MUC1-specific titres, end-point titres are the inverse of the dilution that gave a mean OD_{405nm} higher than the OD_{405nm} obtained with a 1:50 dilution of pooled serum from wild-type CPMV-immunised mice.

Flow cytometry

Tumour cells (410.4, E4 and T47D; $2x10^5$) were incubated with 40 µl of 1:4 diluted antisera (either NMS, or wild type-, CPMV-MUC1(16)- and CPMV-MUC1(23)-immunised serum) or 1:2 diluted monoclonal antibody HMFG2 supernatant for 1 h on ice. After washing with PBS/3%FCS, the cells were incubated with 20 µl of 1:20 diluted PE-labelled goat anti-mouse IgG (Southern Biotechnology Associates, Inc.). The mean fluorescence intensity of the staining was quantified by flow cytometry (FACSCAN; Becton-Dickinson).

Collection of Intestinal IgA

Intestinal lavages were collected from mice as follows. Mice were culled by exsanguination and the intestines and caeca washed out with 3 ml of ice-cold 50 mM EDTA containing soyabean trypsin inhibitor. The lavage fluids were centrifuged at 13000 g for 10 mins to remove large debris and then 10 µl/ml of 0.2M phenylmethyl-sulphonylfluoride (PMSF) in ethanol (95% v/v) and 10 µl/ml of sodium azide (2% w/v) added to the clarified supernatant. Foetal calf serum (FCS; Gibco) was added to 3% and the samples were stored at -80°C.

Example 1 (immunogenicity of MUC1-based CVPs)

Mice (8/group) were immunised subcutaneously with 100 µg of CPMV-MUC1(16), CPMV-MUC1(23) or wild-type CPMV in Freund's complete adjuvant (FCA), Sigma. Subsequent 25 µg boosters in incomplete Freund's adjuvant (IFA) were administered on day 14 and 28, and sera collected on day 42 for analysis by ELISA.

Both CVPs and the wild-type CPMV elicited very high titres of CPMV-specific antibody on day 42 (Figs. 2A-C). However, only the CVP-immunised mice produced MUC1-specific antibody, which was not detected in the sera of wild type immunised mice (Figs. 2A-C). Titres elicited by CPMV-MUC1(16) (mean titre of 100,800) and CPMV-MUC1(23) (mean titre of 86,300) were generally similar.

Example 2 (CPMV-MUC1(16) and CPMV-MUC1(23)-immunised mouse serum stain MUC1-expressing tumour cell lines)

Sera from mice immunised with the above CVPs in FCA (see Example 1) were shown to stain MUC1-expressing mouse E4 and human T47D tumour cells very intensely by flow cytometric analysis (Fig. 3A). The staining of both cell lines using sera from CPMV-MUC1(16)-immunised mice was stronger than that of CPMV-MUC1(23)-immunised sera (Fig. 3A). The staining of T47D cells using sera from CPMV-MUC1(16)-immunised mice was comparable to that achieved with the anti-MUC1 mAb HMFG2.

In a second experiment, sera from mice immunised with CPMV-MUC1(16) in QS-21 also strongly stained the two MUC1-expressing cell lines but not the MUC1-negative 410.4 cells (Fig. 3B). In both studies, the staining was highly specific since the staining of the MUC1-expressing cells with sera from wild-type CPMV-immunised mice and normal mouse serum was extremely weak. Mice immunised with the MUC1 peptide conjugated to KLH elicited only low titres of MUC1-specific IgG and consequently stained the cells only very weakly (Fig. 3B).

The intense specific staining of the cells could also be visualised under a fluroescence microscope. Sera from CPMV-MUC1(16)-immunised mice strongly stained both cell lines, which were only very weakly stained with sera from wild-type CPMV-immunised mice. In contrast, the staining of MUC1-negative 410.4 cells was extremely weak.

Sera from CPMV-MUC1(16) and CPMV-MUC1(23)-immunised mice was also demonstrated to specifically stain fresh human breast cancer tissue (data not shown).

Example 3 (CPMV is a more effective carrier of the MUC1 peptide for the production of specific antibody than is the conventional carrier, KLH).

Mice were immunised subcutaneously with 2 doses of 100 μg CPMV-MUC1 with and without QS-21 on days 0 and 21. Other groups of mice received 2 doses of 10 μg CPMV-MUC1(16) or MUC1-KLH in QS-21 for comparison. Mice immunised with CPMV-MUC1(16) without adjuvant generated significant titres of MUC1-specific antibody (Fig.

4). The generation of MUC1-specific antibody was very rapid as levels were detectable on day 14 (not shown) and had reached high levels 21 days after primary immunisation (Fig. 4). Again, MUC1-KLH was far less effective at generating MUC1-specific IgG even when adjuvant was used. These studies indicate that presenting two 2 μg doses of peptide, as contained in 100 μg of the CVP, without additional adjuvant, leads to high levels of serum antibody which exceed the levels attained using two 100 μg doses of the MUC1 peptide conjugated to KLH using QS-21 as adjuvant. Thus, the CVP presented 50-fold less peptide than the peptide-KLH conjugate and still induced a 50-fold increase in the titre of anti-peptide antibody in the immunised animals.

Example 4 (CPMV is a more effective carrier of the MUC1 peptide for the production of specific antibody than is KLH).

Mice (5 per group) were immunised subcutaneously on days 0 and 14 with 1 or 10 μg of CPMV-MUC1(16) or 50 μg of MUC1 peptide conjugated to KLH. QS-21 was used as an adjuvant in all immunisations. The end-point titres of anti-peptide antibodies on day 21 are shown in the following table.

Immunogen	Mouse No.				
	1	2	3	4	5
10 μg CPMV-MUC1(16)	6400	12800	51200	51200	208400
1 μg CPMV-MUC1(16)	25600	25600	25600	25600	102400
50 μg MUC1-KLH	3200	3200	102400	3200	150

The conclusion is that two doses of 1 μg of CPMV-MUC1(16) (presenting approximately 20ng of the 16-mer peptide per dose) induced generally higher and more consistent titres of anti-peptide antibodies than did two doses of 50 μg of the MUC1 16mer peptide conjugated to KLH. This is particularly remarkable since there is in excess of 2000-fold more peptide being presented by the KLH conjugate.

Example 5 (CPMV is a more effective carrier of the MUC1 sequence than KLH in MUC1-transgenic mice)

Transgenic mice expressing MUC1 and non-transgenic mice were immunised subcutaneously

with 100 μg of CPMV-MUC1(16), wild type CPMV or with 25 μg MUC1 peptide conjugated to KLH (in transgenic mice only) in QuilA and boosted with the same dose on day 14. Sera were pooled for mice in the same group, except for the transgenic mice immunised with CPMV-MUC1(16) which were analysed individually (Fig. 5).

CPMV-MUC1(16) was shown to be highly immunogenic in human MUC1-transgenic mice with anti-MUC1 peptide antibody titres only a little lower than those elicited in non-transgenic mice (Fig. 5). Moreover, the anti-MUC1 peptide antibodies elicited in the transgenic mice by CPMV-MUC1(16) immunisation were considerably greater than those elicited by the MUC1 peptide conjugated to KLH (Fig. 5). The average titres of anti-peptide antibodies were 40,500 following immunisation with CPMV-MUC1(16), 400 following immunisation with MUC1-KLH, and 120 following immunisation with wild type CPMV (see Fig. 5). These data confirm and further support the enhanced presentation system of the present invention by demonstrating that the presentation of a mucin peptide on the surface of a plant virus is significantly better than the presentation as conjugated to KLH (a conventional carrier). In summary, over 10-fold less peptide was presented on CPMV as compared to KLH, and a 100-fold higher level of anti-peptide antibody was induced by CPMV-MUC1(16) as compared to corresponding presentation by KLH (1,000-fold difference in total).

The significance of this study is that the immunised animals were transgenic mice expressing the human MUC1 gene as a self-antigen in the correct tissue similar to that seen in humans. The human mucin gene shows only 30% homology to mouse mucin. Therefore the MUC1 peptide, as derived from human mucin, would be recognized as a foreign antigen in mice but as a self-antigen in humans. In order to develop a mouse model that is more representative of the human situation, transgenic mice expressing human mucin were made (as described in reference 1). It is expected that these transgenic mice will treat the human mucin as a self-antigen, as occurs in humans. These transgenic mice are, therefore, a more realistic model for studying the potential ability of CPMV-MUC1 to induce anti-peptide antibodies in humans.

Example 6 (The relative efficacy of adjuvants to augment the MUC1-specific responses elicited by CPMV-MUC1(16)).

Since FCA is not a suitable adjuvant for human use, further studies were conducted with CPMV-MUC1(16) adjuvanted with less toxic adjuvants including the saponin-based adjuvants QuilA, QS-21 and ISCOM matrix, or with algammulin (a mixture of both alum- and gamma-inulin). Mice were immunised subcutaneously with 50 μg of CPMV-MUC1 in the above adjuvants and boosted with 25 μg on days 14 and 28 with the same adjuvant. Mice immunised with CPMV-MUC1(16) in QS-21 elicited higher titres of MUC1-specific antibody than all other adjuvants tested (Fig. 6), including FCA, when assayed on day 42. Titres in all the adjuvant groups remained high for 70 days and then began to drop, except for the FCA and QS-21 groups which still had high titres on day 150 (mean titres of 76,800 and 57,600 respectively; Fig. 6).

Example 7 (Adjuvant is not required to elicit a good immune response to CVPs of the present invention)

Mice were immunised subcutaneously with 100 μg of CPMV-MUC1(16) on days 0 and 21 with QS-21 and on days 0 and 14 without QS-21 as adjuvant. On day 37, the titres of the anti-MUC1 peptide antibodies were not very different between the mice immunised with or without adjuvant (see Fig. 4). Although the use of QS-21 is beneficial in increasing, and probably also prolonging, the immune response to the mucin peptide present on the CPMV particle, the use of an adjuvant such as QS-21 is not essential. CPMV-MUC1(16) administered without any adjuvant induced a good immune response to the MUC1 peptide.

Example 8 (CVPs are potent simulators of systemic and mucosal immune response following intranasal vaccination)

Mice were immunised intranasally on days 0, 7, 14, and 21 with 100 μg per dose of CPMV-MUC1(16) or wild-type CPMV. The immunisations were carried out with or without the use of cholera toxin (CT). CT has an adjuvant activity via its ability to recognise certain cell-surface receptors and thus aid in the delivery of antigen to professional antigen presenting cells.

The titres of anti-peptide IgG antibodies in the sera following intranasal administration of CPMV-MUC1(16) were approximately the same when the CVP was administered with or without CT (1:53,440 with CT, 1:57,680 without CT, see Fig. 7A). In addition, high titres

of anti-MUC1 IgA antibodies in intestinal lavages were found in animals immunised with or without CT (see Fig. 7B).

The conclusion is that CT is not necessary to induce mucosal or systemic immune responses to CPMV-MUC1(16) administered via a nasal route. Furthermore, CT does not enhance the immune response to CPMV-MUC1(16) following intranasal administration.

Example 9 (Vaccination with CPMV-MUC1(16) causes a reduction in tumour burden in mice tumour protection studies)

The ability of CPMV-MUC1(16) to elicit antibodies which can cause regression of tumours expressing the MUC1 protein was demonstrated in a mouse tumour model. Tumour cells expressing the MUC1 protein were injected subcutaneously into mice which had previously been immunised with CPMV-MUC1(16). Full details are described below.

Nine C57BLScSn mice were immunised subcutaneously with 100 μg of CPMV-MUC1(16) on day 0 and 21 using Quil A (10 mg/dose) as adjuvant. A further 6 mice were immunised with phosphate buffered saline (PBS) containing Quil A (PBSA) in a similar manner. On day 42 all mice were administered with 2 x 10^5 MUC1 RMA cells by subcutaneous injection in the flank and the tumour size measured in two dimensions over the following month using vernier calipers. Tumour volumes were calculated as $(a \times b^2)/2$ where a represents the largest diameter and b the smallest diameter. Mice were sacrificed when tumours reached $\sim$1 to 1.5 cm in diameter. The results are summarised below:

		Days post challenge						
Mouse No.	Vaccine	9	14	16	21	23	28	30
1	CPMV-MUC(16)	0.108	0.06	0	0	0	0	0
2	CPMV-MUC(16)	0.0108	0.06	0.032	0.032	0.032	0.013	0.004
3	CPMV-MUC(16)	0.05	0.05	0	0	0	0	0
4	CPMV-MUC(16)	0.062	0.032	0	0	0	0	0
5	CPMV-MUC(16)	0.062	0.062	0.062	0.0864	1.14	1.8	
6	CPMV-MUC(16)	0.108	0.126	0.196	0.6	0.85	1.617	
7	CPMV-MUC(16)	0.126	0.126	0.126	0.441	0.793	1.68	
8	CPMV-MUC(16)	0.126	0.126	0.108	0	0	0	
9	CPMV-MUC(16)	0.075	0.148	0.22	1.27			
10	PBSA	0.126	0.5	0.55	1.68			
11	PBSA	0.108	0.428	0.5	1.37			
12	PBSA	0.17	0.256	0.256	0.936			
13	PBSA	0.075	0.5	0.55	1.37			
14	PBSA	0.0907	0.108	0.108	0.256	0.5	1.372	
15	PBSA	0.171	0.5	0.726	1.47			

It can been seen from the above data that over 50% of the mice (5/9 mice) immunised with CPMV-MUC1 were protected compared to 0% (6/6) in the PBSA-immunised control

group.

Whereas Examples 1-9 (above) illustrate presentation of a tumour-associated mucin from the S subunit (ßB-ßC insertion site) of CPMV, alternative insertion sites within the S subunit are suitable for achieving the CVPs of the present invention. In this respect, knowing the nucleic acid sequence encoding the S subunit and a range of desired insertion locations therein (see pages 7-11), the skilled person would simply prepare a suitable expression vector in a manner analogous to the preparation of pCP7.

Similarly, knowing the nucleic acid sequence encoding the L subunit of CPMV and a range of desired insertion locations therein (see pages 7-11), the skilled person would simply prepare an expression vector encoding, *inter alia*, the L subunit in a manner analogous to the preparation of pCP7.

Although the above examples employ the MUC1(16) or MUC1(23) epitopes, any mucin peptide epitope sequence can be used.

The following Examples illustrate the application of the present invention to plant viruses other than CPMV. In addition, any icosahedral plant virus can potentially be used as a carrier for these mucin peptides.

Example 10

This example describes the use of a plant virus other than CPMV, whose crystal structure is known, as a carrier for the MUC1(16) epitope described previously.

Inspection of the crystal structure of Southern bean mosaic virus (SBMV) strain C reveals that a portion of the loop between the βH and βI strands is well exposed upon the surface of the virus at the five-fold and quasi-six fold axes. This portion of the loop comprises amino-acids 251 to 255 of the linear coat protein sequence and nucleotides 3967 to 3981 of the genomic RNA sequence.

The cDNA of the complete 4194 bp RNA genome of SBMV is cloned into a derivative of pBluescript II plasmid vector lacking the T7 and T3 promoters using standard molecular biological techniques. The cDNA is cloned immediately downstream of a bacteriophage T7 such that a unique restriction enzyme site is present at the 3' terminus of the cDNA, thus allowing linearisation of the recombinant plasmid to generate run-off transcripts which mimic the wild-type RNA. As an alternative, the cauliflower mosaic virus (CaMV) 35S promoter may be used.

A sub-clone is then made from this full-length cDNA clone by inserting the *Bgl*II to *Xmn*I fragment (genomic RNA nucleotides 3165 to 4161), which contains within it the whole coat protein open reading frame, into *Bam*HI/*Hinc*II digested pBluescript II. This sub-clone is further manipulated via site-directed mutagenesis at genomic nucleotide positions 3969 (change A to C) and 3984 (G to T) to create *Bam*HI and *Hpa*I restriction sites, respectively.

The modified subclone is digested with these enzymes and separated from the small excised fragment which is replaced by oligonucleotides coding for the excised nucleotide sequence plus nucleotides coding for the epitope sequence MUC1(16). The following five constructs contain the inserted peptide sequence either between coat protein amino-acids 251 and 252, or 252 and 253, or 253 and 254, or 254 and 255, or 255 and 256 (see Fig. 8).

The modified region of the coat protein from each of these constructs is isolated on a *Hind*III/*Avr*II fragment (genomic nucleotides 3434 to 4096) and used to replace the corresponding fragment in the full-length cDNA clone of the virus. Each of these clones is then linearised at the 3' terminus of the cDNA and, in the case of a T7 bacteriophage promoter construct, used to generate capped run-off RNA transcripts which are then inoculated onto the host-plant (*Vigna unguiculata*), or inoculated directly when under the control of the 35S promoter.

The inoculated plants are monitored for symptoms, and the strength of symptoms, yield and stability for each construct are assessed in order to determine the optimal insertion site. If desirable, purified virus may also be used to immunise experimental animals in order to determine the levels of immune response generated by each construct.

This Example can be extrapolated to allow insertion in any of the exposed loops of SBMV. Similary, any mucin peptide epitope sequence can be used instead of MUC1(16).

Example 11

This example describes the determination of a potential insertion site for epitopes by alignment of the primary sequence of a virus whose structure is unknown (lucerne transient streak virus, LTSV), against those of viruses whose structure has been determined.

The crystal structures of two sobemoviruses, SBMV and Sesbania mosaic virus (SMV), have been solved at high resolution. Comparison of the crystal structures reveals that all the secondary structural elements are well conserved between the viruses and, in particular, the protruding loop between the βH and βI is almost identical in shape and location between the two viruses. This structural element would therefore be expected to be well conserved in all sobemoviruses.

Alignment of the primary sequences of LTSV, SBMV and SMV shows a strong conservation of residues between the three viruses within the βH strand region and significant sequence homology within the βI strand (see Fig. 9). This allows the loop region of LTSV to be inferred as spanning amino acids 218 to 224 of the coat protein.

The 4.275 kb RNA genome is cloned as cDNA, as described for SBMV in Example 10. The genomic clone is then modified by site directed mutagenesis at position 3959 (C to T) and position 3998 (T to C) to create unique *Pst*I and *Kpn*I restriction enzyme sites, respectively. The modified genomic clone is digested with these restriction enzymes and separated from the small excised fragment which is replaced by oligonucleotides coding for the excised nucleotide sequence plus nucleotides coding for the epitope sequence MUC1(16). The following six constructs contain the epitope sequence either between coat protein amino acids 218 and 219, or 219 and 220, or 220 and 221, or 221 and 222, or 221 and 223, or 223 and 224 (see Fig. 10).

Each of these clones is linearised at the 3' terminus of the cDNA and, in the case of a T7 bacteriophage promoter construct, used to generate capped run-off RNA transcripts which are then inoculated onto the host-plant (*Nicotiana clevelandii*), or inoculated directly when under the control of the 35S promoter.

The inoculated plants are monitored for symptoms, and the strength of symptoms, yield and stability for each construct are assessed in order to determine the optimal insertion site. If desirable, purified virus may also be used to immunise experimental animals in order to determine the levels of immune response generated by each construct.

This Example can be extrapolated to allow insertions in any of the exposed loops of LTSV. Similarly, any mucin peptide epitope sequence can be used instead of MUC1(16).

Example 12

This example describes the determination of potential insertion sites in a virus (red clover necrotic mosaic virus, RCNMV), whose crystal structure is unknown, using primary structural alignments with a second virus whose crystal structure has been determined (tomato bushy stunt virus, TBSV) in conjunction with secondary structure prediction algorithms

The crystal structure of the coat protein of TBSV reveals that each of the 180 coat protein subunits forming the T=3 icosahedron consists of two β-barrel domains. The first domain forms the surface of the virus particle and is termed the S domain and is equivalent to the single domain found in SBMV. The second, much smaller, domain forms a surface protrusion at right angles to the plane of the S domain. This P domain forms dimeric interactions with the P domain of a neighbouring coat-protein subunit at the strict and quasi two-fold axes of the icosahedron. The presence of the P domain causes the virions to appear distinctly granular when examined under the electron microscope. Between the S and P domains is a short flexible linker followed by a pair of β-strands connected by a loop which appears to be highly exposed on the viral surface with no obvious role in the contacts between subunits. This loop provides a potential target for epitope insertions.

Dianthoviruses (e.g. RCNMV) also appear distinctly granular when subjected to electron microscopy, and this together with the size of the coat proteins and their limited homology with those of tombusviruses suggests that they may have structural similarity. Alignment of the coat protein sequences of RCNMV and TBSV (see Fig. 11) using a Lipman-Pearson alignment algorithm, which recognises sequence conservation as well as identity, gives a similarity index of 26.9 (strict homology is 23%). From the alignment it can be seen that the S domain is better conserved (TBSV residues 100 to 269, strict homology 36%) than the P domain (TBSV residues 270-388), which is poorly conserved.

The loop of interest comprises TBSV residues L^{280} A^{281} G^{282} and the sequence around this region shows some similarity to the sequence of RCNMV, however secondary structure prediction algorithms are also used to predict the location of β-strands and hence the loops which lie between them. Fig. 12 shows a Chou-Fasman β-region prediction plot of RCNMV residues 214-257 using an algorithm based upon the structures found in 64 proteins. This type of plot is claimed to be 80% accurate at predicting beta strands of interest. The plot suggests that beta strands of interest are located between residues 214-221 and 226-228, hence the loop at the tip of the domain will be residues 222-225 and residues 245-248. A more sophisticated prediction algorithm, the EMBL PHDsec program based upon trained neural networks, may also be used. The resulting output for the region of interest is shown in Fig. 13. This locates the β-strands to residues 220-223 and 227-239, therefore the loop is comprised of residues 224-226. Combining the two sets of data, the loop will lie within the region spanned by residues 222 to 226.

Dianthoviruses have a bipartite RNA genome, both RNAs being required for infectivity. Accordingly, RCNMV RNAs 1 and 2 are cloned as cDNA, using standard molecular biological techniques, into a suitable vector, downstream of a CaMV 35S promoter. As an alternative, the T7 promoter may be used. Both clones are engineered such that they can be linearised at the 3' termini of the cDNAs.

The cDNA genomic clone of RNA1 is modified by site directed mutagenesis at positions 3078 (A to G) and 3081 (G to C), to create a unique *Apa*LI restriction site, and at positions

3108 to 3111 (ACTC to GTTA) to create a unique *Hpa*I restriction site. The mutation at position 3081 is not silent, however the correct codon is restored when ligating in oligonucleotides to generate the epitope insertion. The modified genomic clone is digested with these restriction enzymes and separated from the small excised fragment which is replaced by oligonucleotides coding for the excised nucleotide sequence plus nucleotides coding for the epitope sequence MUC1(16). The following six constructs, contain the epitope sequence either between coat protein amino acids 221 and 222, or 222 and 223, or 223 and 224, or 224 and 225, or 225 and 226, or 226 and 227 (see Fig. 14).

Each of these clones is linearised at the 3' terminus of the cDNA and, in the case of a T7 bacteriophage promoter construct, used to generate capped run-off RNA transcripts. These are then mixed with similar transcripts from the linearised cDNA clone of genomic RNA2 and inoculated onto the host-plant (*Nicotiana clevelandii*). Linearised clones are inoculated directly when under the control of the CaMV 35S promoter.

The inoculated plants are monitored for symptoms, and the strength of symptoms, yield and stability for each construct are assessed in order to determine the optimal insertion site. Purified virus may also be used to immunise experimental animals in order to determine the levels of immune response generated by each construct.

This Example can be extrapolated to allow insertions in any exposed loops of RCNMV. Similarly, any mucin peptide epitope sequence can be used instead of MUC1(16)

Summary of SEQ. ID's :

SEQ. ID NO. 1 = "PDTRPAPGSTAPPAHGVTSA"

SEQ. ID NO. 2 = "PDTRP"

SEQ. ID NO. 3 = "APDTR"

SEQ ID NO. 4 = "DAHWESWL"

SEQ ID NO. 5 = "DLHWASWV"

Further Insertion Points in VP-S of CPMV

As mentioned above, the mucin peptide, e.g. the 16-mer, can be inserted in the $\beta C'\beta C''$ loop of the small coat protein of CPMV. For example, the MUC peptide can be cloned in between $D_{44}D_{45}$, in a suitable vector. This results in a good viral yield with good immunological properties. Moreover, insertions in the $\beta C'\beta C''$ loop can be combined with insertions in the C-terminus of the VP-S.

The C-terminus of VP-S sticks out from the surface of the virions and is highly immunogenic. The region that is no part of the "body" of the virus starts with Pro_{182} and runs up to the C-terminus, Ala_{213}. Insertions of MUC epitopes can be made in this region. A particularly effective insertion site is in between $R_{199}S_{200}$.

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CLAIMS

1. A chimaeric virus particle derived from a plant virus having a coat protein with a beta barrel structure and modified by insertion of an immunogenically active peptide of a tumour-associated mucin at an immunogenically effective site in the coat protein.

2. A chimaeric virus particle according to Claim 1, in which the insert is present in a loop connecting beta sheets.

3. A chimaeric virus particle according to Claim 1, in which the insert is present in the region of the C-terminus of a coat protein.

4. A chimaeric virus particle according to Claim 3, in which the insert is present at a point within 30 amino acids, preferably within 15 amino acids, of the C-terminus.

5. A chimaeric virus particle according to any of Claims 1 to 4, in which the tumour-associated mucin is PEM.

6. A chimaeric virus particle according to Claim 5, in which the insert is a peptide derived from the 20 amino acid repeat of the extracellular portion of the MUC1 transmembrane molecule.

7. A chimaeric virus particle according to Claim 6, in which the peptide is a 16-mer or 23-mer peptide.

8. A chimaeric virus particle according to any of the preceding claims, in which the plant virus is a comovirus.

9. A chimaeric virus particle according to Claim 8, in which the plant virus is cowpea mosaic virus.

10. A chimaeric virus particle according to Claim 9, in which the insert is present in the S protein of the virus.

11. A method of producing a chimaeric virus particle according to any of Claims 1 to 10, which comprises introducing a nucleotide sequence coding for the tumour-associated mucin peptide to modify the plant viral nucleic acid which codes for the coat protein; infecting plants, plant tissue, plant cells, or protoplasts with the modified viral nucleic acid; and harvesting chimaeric virus particles.

12. A method according to Claim 11, in which the introduced nucleotide sequence is inserted in that part of the plant viral nucleic acid which codes for an exposed region of the coat protein.

13. A method according to Claim 11, applied to an RNA plant virus, which comprises introducing a DNA coding for the tumour-associated mucin peptide into a cDNA corresponding to the RNA of the plant virus which codes for an exposed portion of its coat protein; inoculating plants, plant tissue, plant cells, or protoplasts with the thus modified cDNA or an RNA transcript thereof, if necessary together with any other DNA or RNA required for multiplication and assembly of whole virus particles in the plant material; and harvesting chimaeric virus particles.

14. A method according to Claim 13, in which the modified cDNA is produced by introducing the DNA encoding the mucin peptide into a DNA fragment excised from the plant viral cDNA, and reinserting the modified excised fragment so as to constitute the plant viral cDNA in modified form.

15. A method according to any of Claims 11 to 14, in which modified virus produced, or RNA extracted therefrom, is passaged in plants to produce further yields of modified virus.

16. A vaccine comprising chimaeric virus particles according to any of Claims 1 to 10 as an immunogenic component thereof.

17. A vaccine according to Claim 16, further comprising an adjuvant.

18. A vaccine according to Claim 17, in which the adjuvant is selected from Freund's complete adjuvant, QuilA, QS-21, ISCOM matrix, algammulin; or combinations thereof.

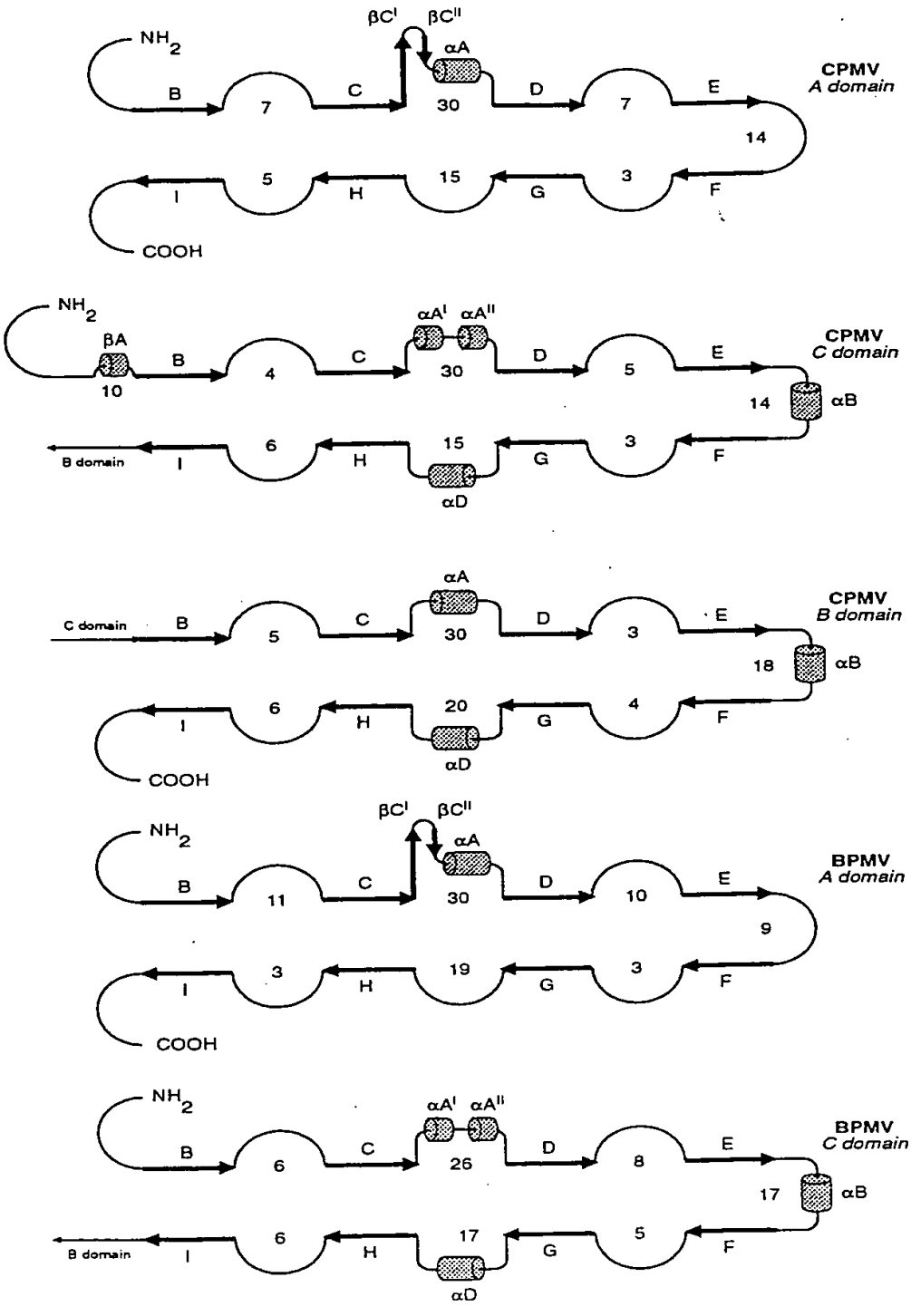
19. A vaccine according to Claim 16, said vaccine being substantially free from adjuvant.

20. A method of eliciting in an animal, including a mammal, an immune response characterised by the production of serum immunoglobulins specific for mucin peptides or polypeptides, which comprises the administration of an immunogenic complex containing chimaeric virus particles according to any of Claims 1 to 10.

ABSTRACT

Modified Plant Viruses

The invention relates to chimaeric virus particles derived from a plant virus having a coat protein with a beta barrel structure and modified by insertion of an immunogenically active peptide of a tumour-associated mucin at an immunogenically effective site in the coat protein, and a method of producing such chimaeric virus particles. The invention also relates to vaccines comprising such chimaeric virus particles as an immunogenic component thereof.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βC^I βC^II
αA
B
7
C
30
D
7
E
CPMV
A domain
14
5
15
3
I
H
G
F
COOH
NH2
αA^I αA^II
βA
B
4
C
30
D
5
E
CPMV
C domain
10
14
αB
B domain
I
6
H
15
G
3
F
αD
αA
C domain
B
5
C
30
D
3
E
CPMV
B domain
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αB
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20
4
I
H
G
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αD
COOH
NH2
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B
11
C
30
D
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E
BPMV
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3
19
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F
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NH2
αA^I αA^II
B
6
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I
6
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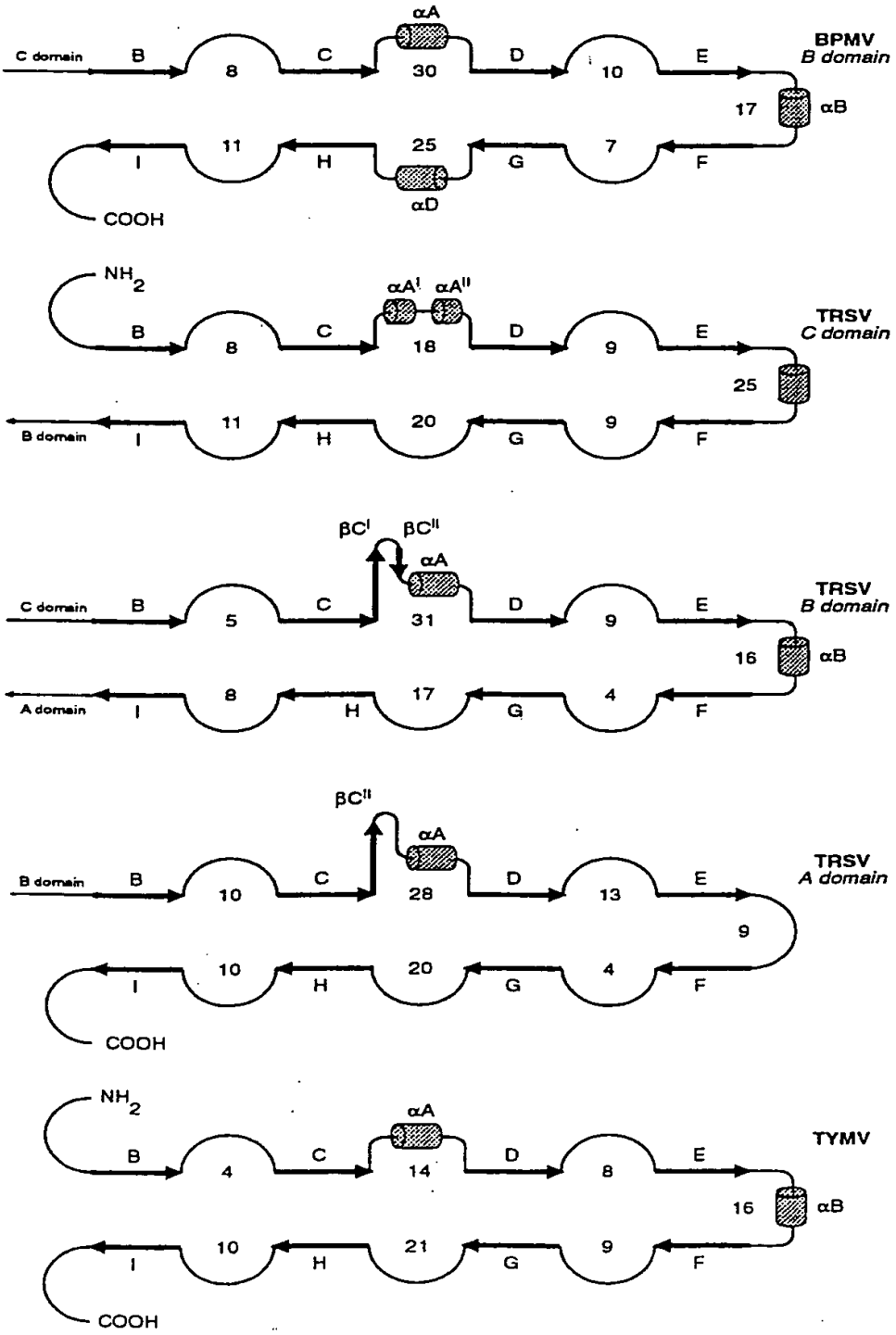
αD

Figure 1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B
8
C
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H
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7
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αD
NH2
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B
8
C
18
D
9
E
TRSV
C domain
25
B domain
11
I
H
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9
F
βC! βC!!
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B domain
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8
I
H
17
G
4
F
βC!!
αA
B domain
B
10
C
28
D
13
E
TRSV
A domain
9
10
I
H
20
G
4
F
COOH
NH2
αA
B
4
C
14
D
8
E
TYMV
16
αB
10
I
H
21
G
9
F
COOH

Figure 1

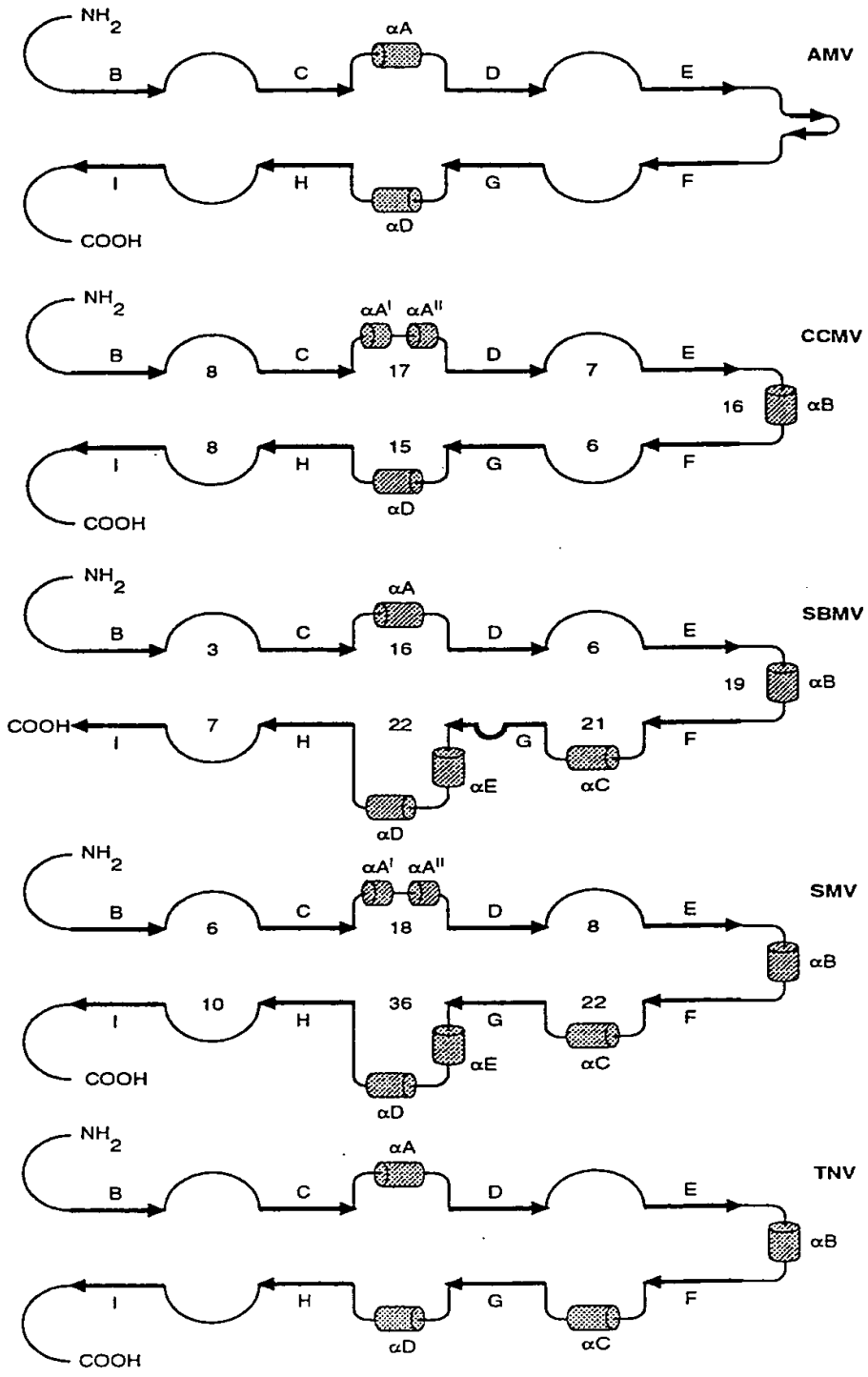
NH2
αA
AMV
B
C
D
E
I
H
G
F
αD
COOH
NH2
αA' αA''
CCMV
B
8
C
17
D
7
E
16
αB
8
15
6
I
H
G
F
αD
COOH
NH2
αA
SBMV
B
3
C
16
D
6
E
19
αB
COOH
7
22
21
I
H
G
F
αE
αC
αD
NH2
αA' αA''
SMV
B
6
C
18
D
8
E
αB
10
36
22
I
H
G
F
αE
αC
COOH
αD
NH2
αA
TNV
B
C
D
E
αB
I
H
G
F
αD
αC
COOH

Figure 1

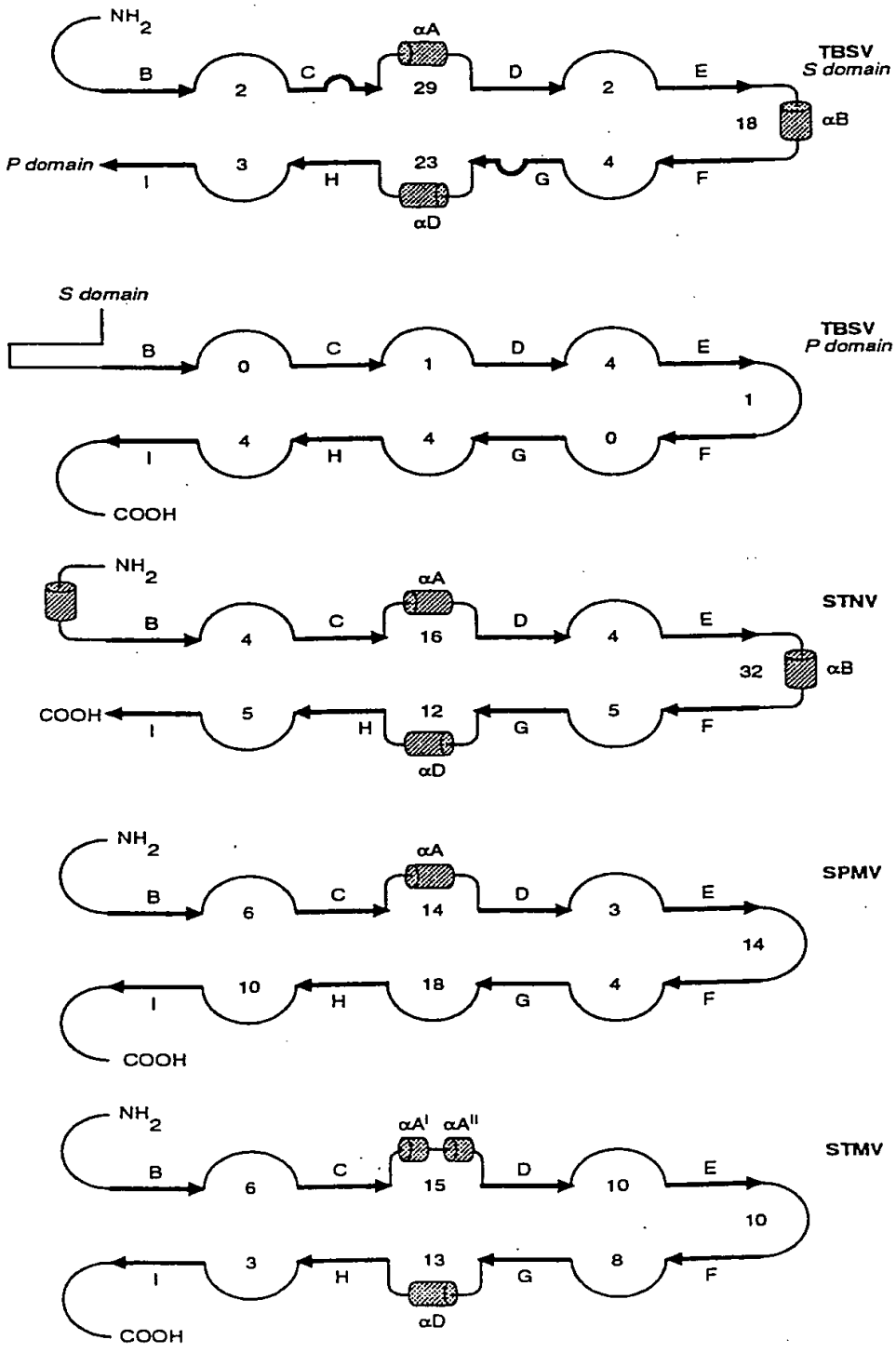
NH2
αA
B
2
C
29
D
2
E
TBSV
S domain
18
αB
P domain
3
23
4
I
H
G
F
αD
S domain
TBSV
P domain
B
0
C
1
D
4
E
1
4
4
0
I
H
G
F
COOH
NH2
αA
STNV
B
4
C
16
D
4
E
32
αB
COOH
5
12
5
I
H
G
F
αD
NH2
αA
SPMV
B
6
C
14
D
3
E
14
10
18
4
I
H
G
F
COOH
NH2
αA^I
αA^II
STMV
B
6
C
15
D
10
E
10
3
13
8
I
H
G
F
αD
COOH

Figure 1

Fig. 2A

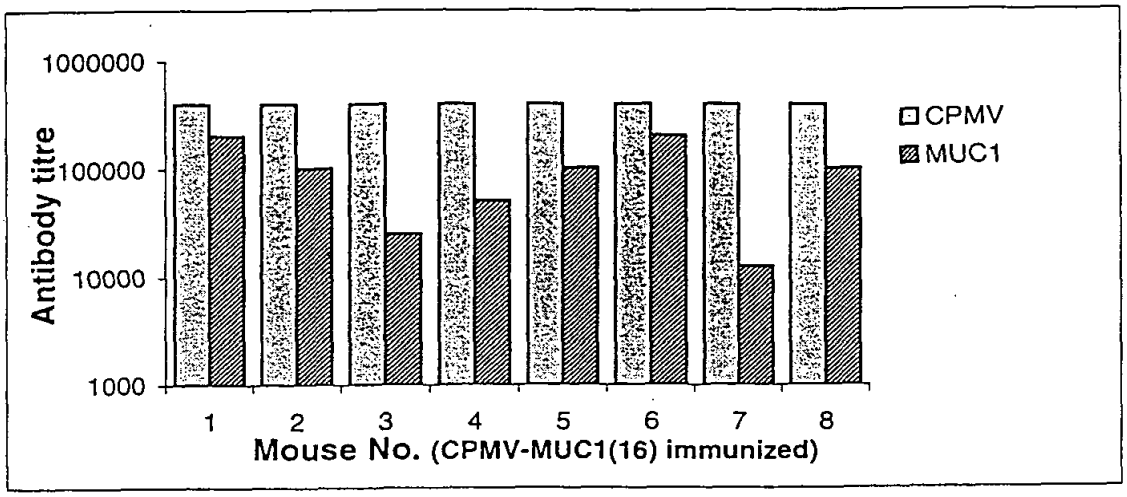

Fig. 2B

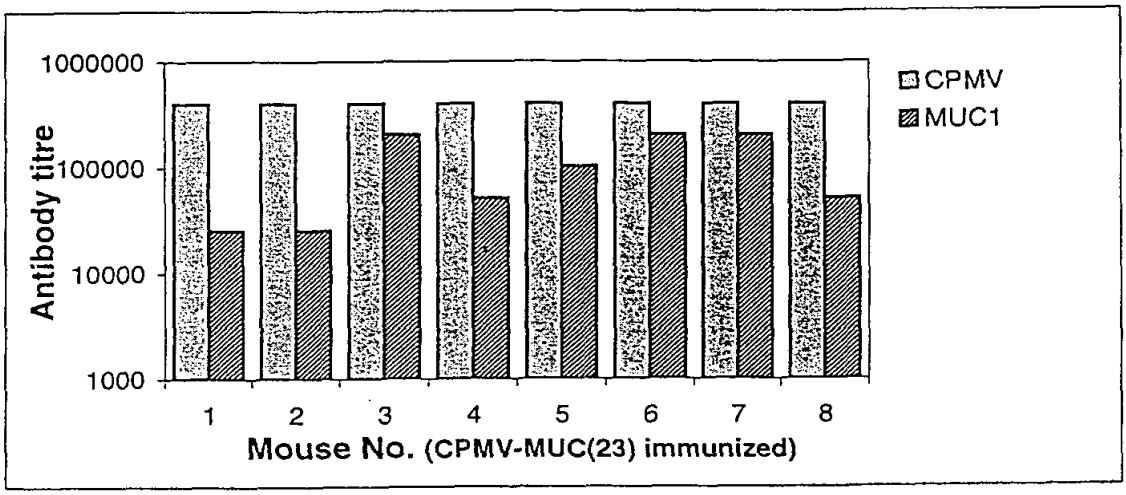

Fig. 2C

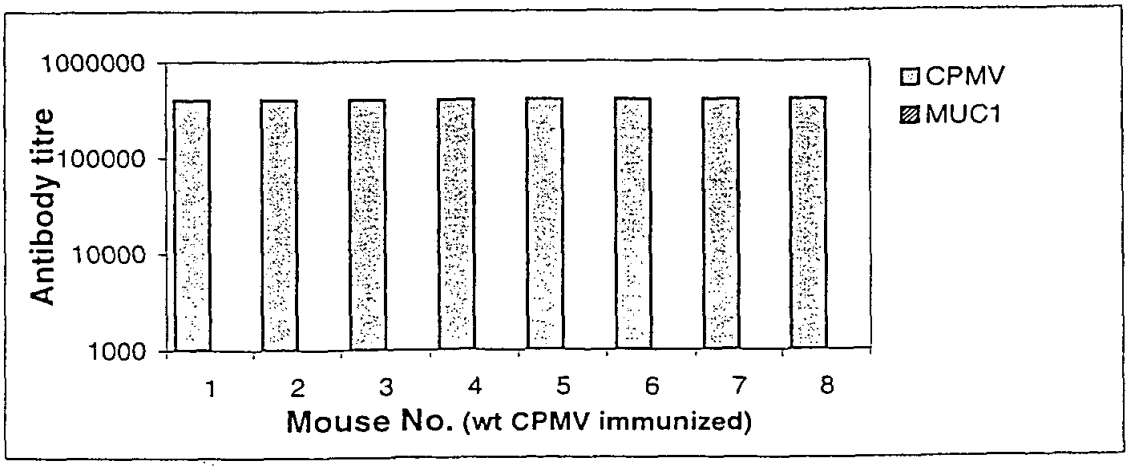

Fig. 3A

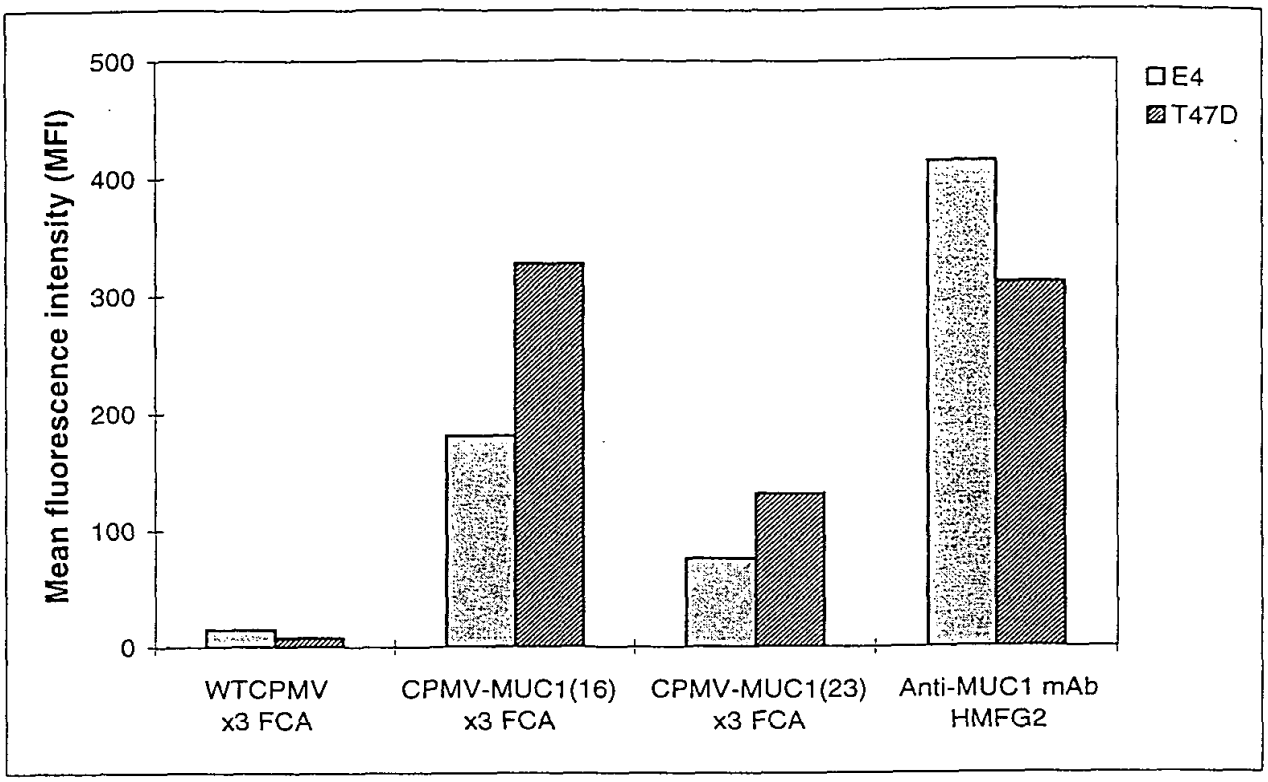
Mean fluorescence intensity (MFI)
500
400
300
200
100
0
E4
T47D
WTCPMV x3 FCA
CPMV-MUC1(16) x3 FCA
CPMV-MUC1(23) x3 FCA
Anti-MUC1 mAb HMFG2

Fig. 3B

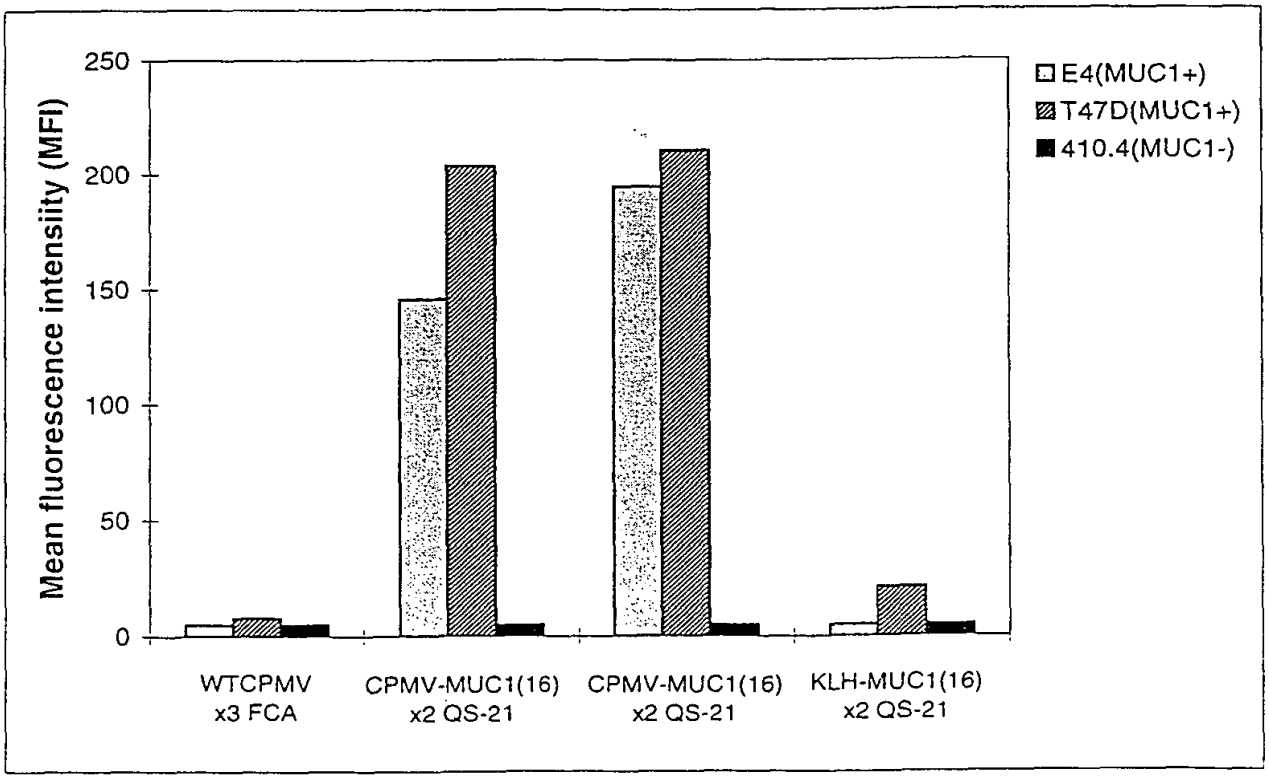
Mean fluorescence intensiity (MFI)
250
200
150
100
50
0
E4(MUC1+)
T47D(MUC1+)
410.4(MUC1-)
WTCPMV x3 FCA
CPMV-MUC1(16) x2 QS-21
CPMV-MUC1(16) x2 QS-21
KLH-MUC1(16) x2 QS-21

Fig. 4

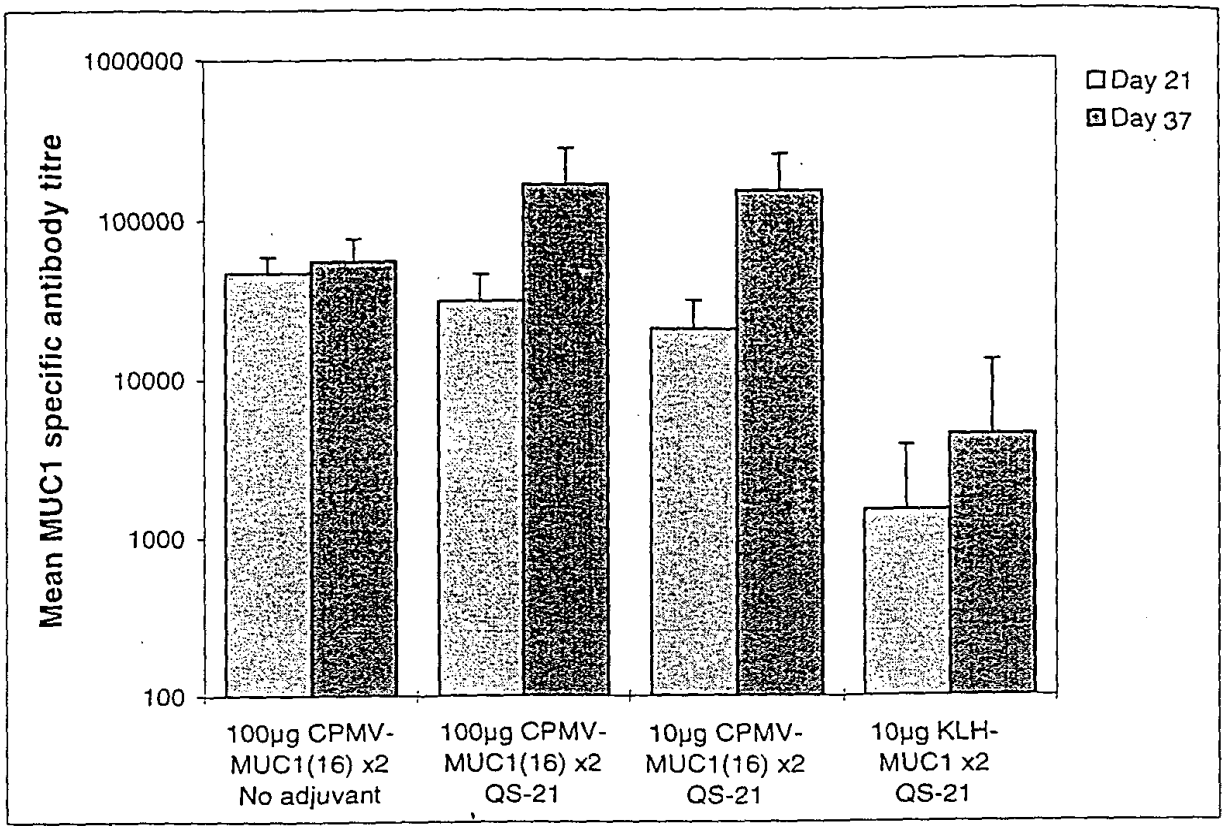
Mean MUC1 specific antibody titre
1000000
100000
10000
1000
100
Day 21
Day 37
100µg CPMV-MUC1(16) x2 No adjuvant
100µg CPMV-MUC1(16) x2 QS-21
10µg CPMV-MUC1(16) x2 QS-21
10µg KLH-MUC1 x2 QS-21

Fig. 5

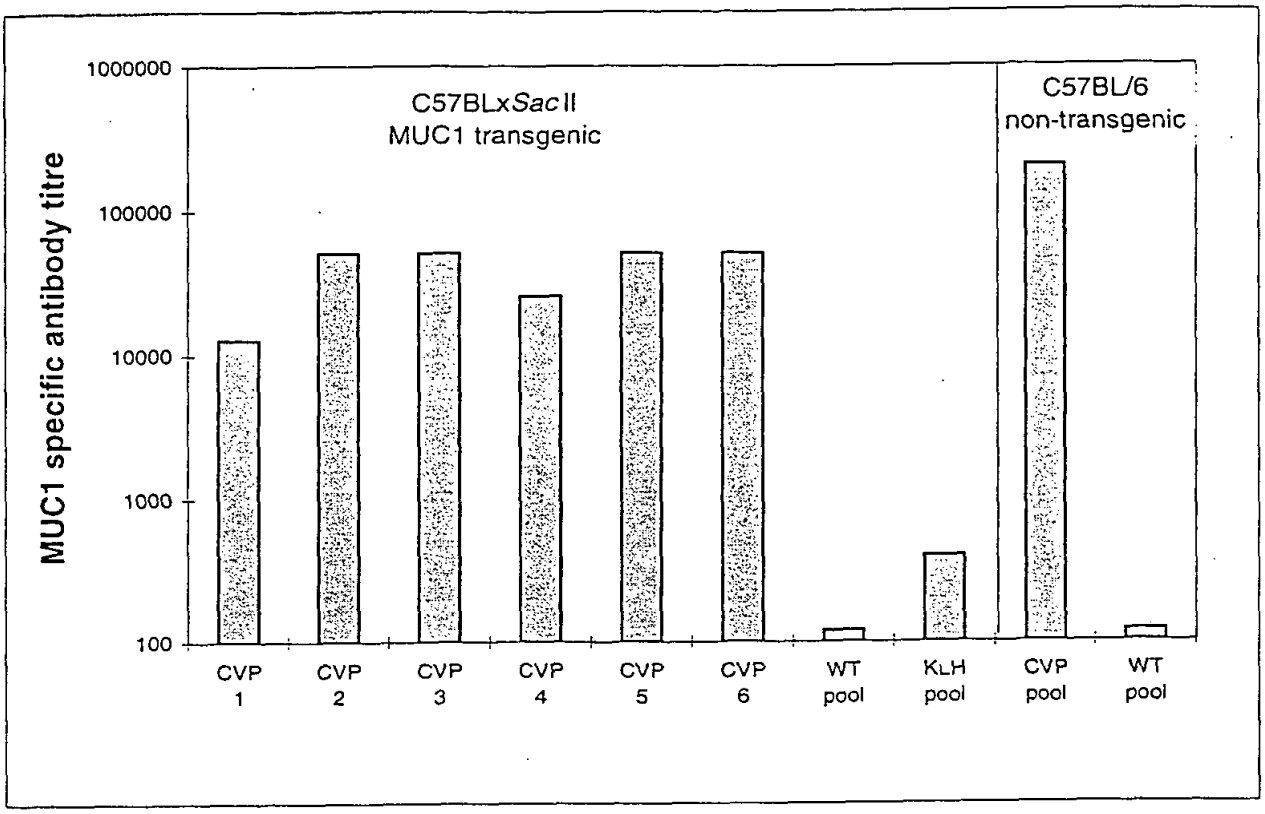
MUC1 specific antibody titre
1000000
100000
10000
1000
100
C57BLxSac II
MUC1 transgenic
C57BL/6
non-transgenic
CVP 1
CVP 2
CVP 3
CVP 4
CVP 5
CVP 6
WT pool
KLH pool
CVP pool
WT pool

Fig. 6

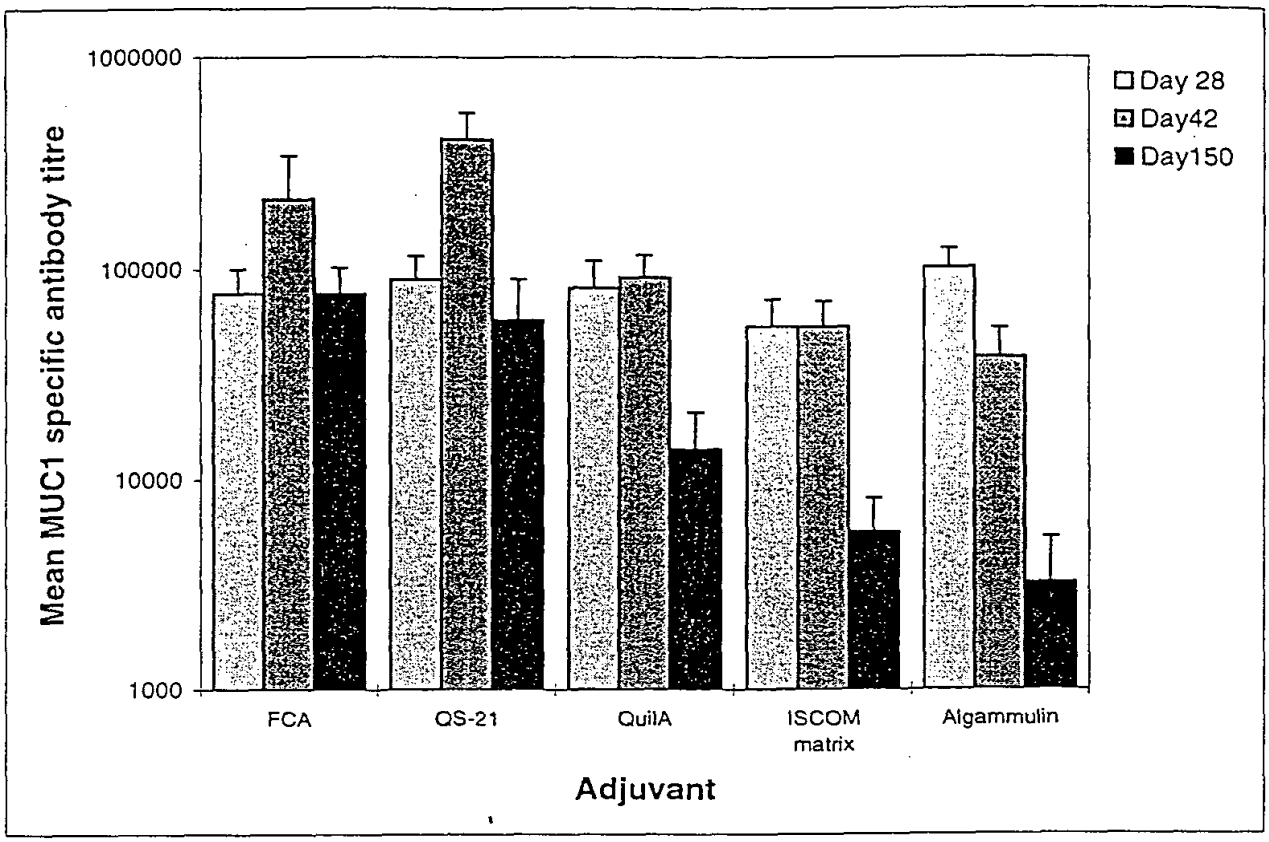
1000000
100000
10000
1000
Mean MUC1 specific antibody titre
Day 28
Day42
Day150
FCA
QS-21
QuilA
ISCOM matrix
Algammulin
Adjuvant

Fig. 7A

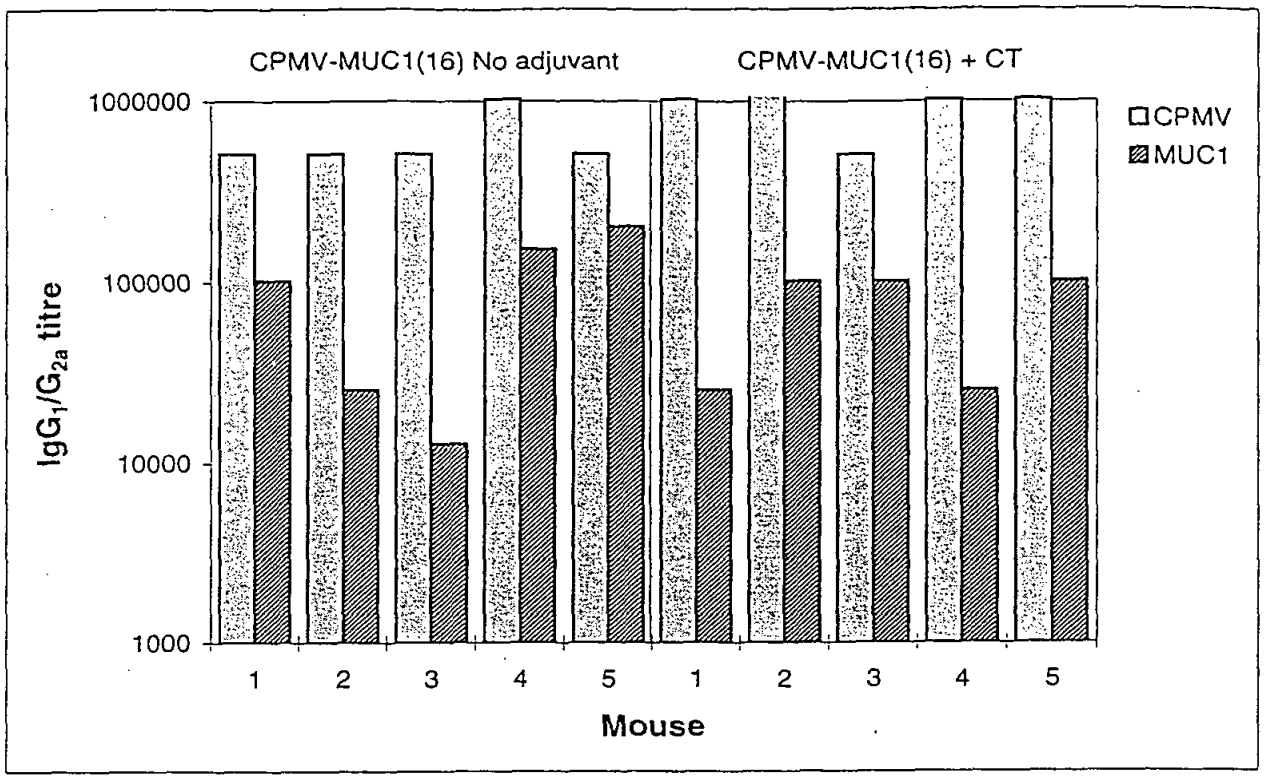
CPMV-MUC1(16) No adjuvant
CPMV-MUC1(16) + CT
1000000
100000
10000
1000
IgG1/G2a titre
CPMV
MUC1
1
2
3
4
5
1
2
3
4
5
Mouse

Fig. 7B

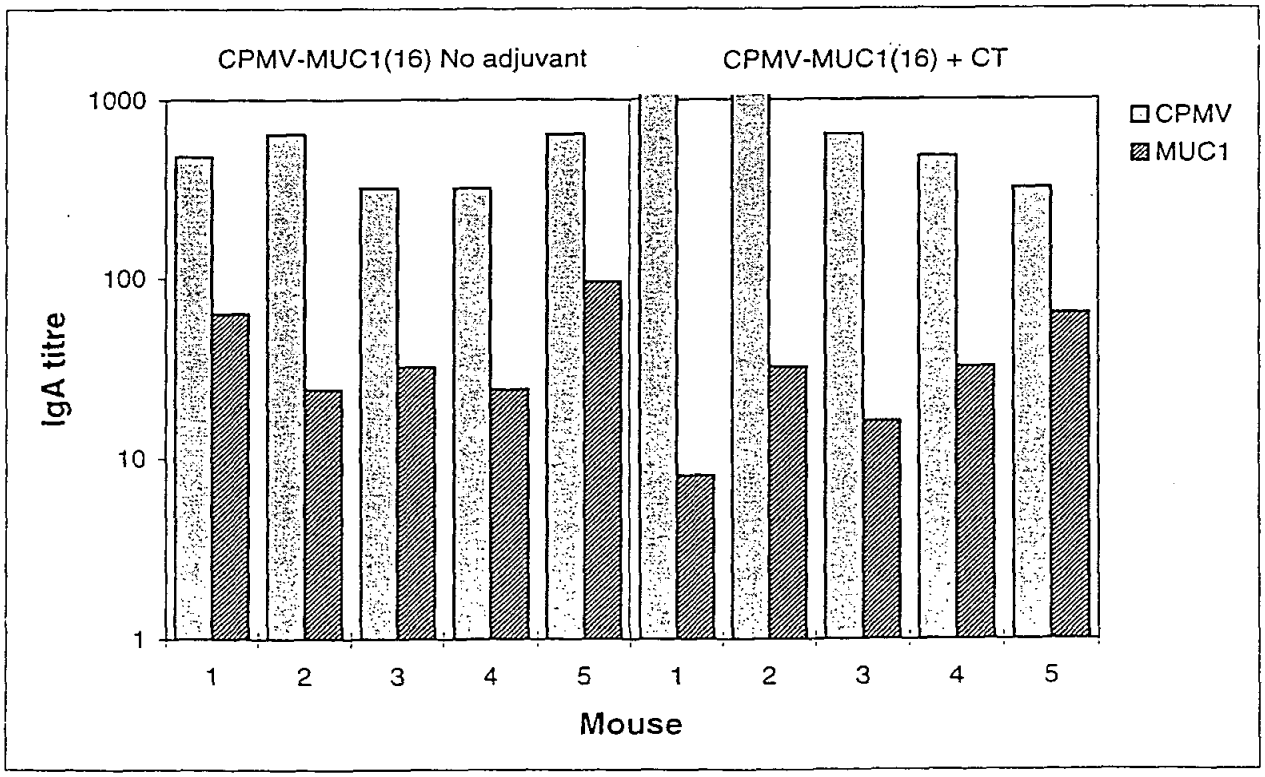
CPMV-MUC1(16) No adjuvant
CPMV-MUC1(16) + CT
1000
100
10
1
IgA titre
CPMV
MUC1
1
2
3
4
5
1
2
3
4
5
Mouse

Figure 8.

11/17

(a) Sequence of SBMV Coat Protein Spanning The Potential Insertion Site With Introduced Base Changes and New Restriction Sites: (sequence starts at nt 3955)

```
 M  E  G  G  S  S  K  T  A  V  N  T  G
ATGGAAGGAGGATCATCTAAGACTGCTGTGAACACTGGG
              ↓                 ↓
           GGATCC            GTTAAC
           BamH I            Hpa I
```

(b) Series of Sequences to be Inserted Between the Restriction Sites to Insert the MUC1(16) Epitope at Various Locations

```
 G  V  T  S  A  P  D  T  R  P  A  P  G  S  T  A
GGTGTTACTTCTGCTCCTGATACTAGACCTGCTCCTGGTTCTACTGCT
CCACAATGAAGACGACCACTATGATCTGGACGAGGACCAAGATGACGA
```

GATCC	TCTAAGACTGCTGTT
G	AGATTCTGACGACAA
GATCCTCT	AAGACTGCTGTT
GAGA	TTCTGACGACAA
GATCCTCTAAG	ACTGCTGTT
GAGATTC	TGACGACAA
GATCCTCTAAGACT	GCTGTT
GAGATTCTGA	CGACAA
GATCCTCTAAGACTGCT	GTT
GAGATTCTGACGA	CAA

Figure 9.

```
LTSV  : NI---YAPARLTIAA-ANSSINIASVGTLYATYEVEL
SBMV  : NIGNILVPARLVIAMEGGSSKTAVNTGRLYASYTIRL
SMV   : NIATDLVPARLVIALLDGSSSTAVAAGRIYASYTIQM
                  #######=======##############
                     βH     loop         βI
```

Figure 10.

(a) Sequence of LTSV Coat Protein Spanning The Potential Insertion Site With Introduced Base Changes and New Restriction Sites: (sequence starts at nt 3954)

```
I  A  A  A  N  S  S  I  N  I  A  S  V  G  T  L  Y
ATAGCCGCAGCTAACAGCTCCATAAACATAGCTAGTGTGGGTACTCTTTAT
     ↓                                       ↓
     CTGCAG                                  GGTACC
     PstI                                    KpnI
```

(b) Series of Sequences to be Inserted Between the Restriction Sites to Insert the MUC1(16) Epitope at Various Locations.

```
G  V  T  S  A  P  D  T  R  P  A  P  G  S  T  A
GGTGTTACTTCTGCTCCTGATACTAGACCTGCTCCTGGTTCTACTGCT
CCACAATGAAGACGACCACTATGATCTGGACGAGGACCAAGATGACGA
```

GCTAACAGC	TCCATAAACATAGCTAGTGTGGGTAC
ACGTCGATTGTCG	AGGTATTTGTATCGATCACACC
GCTAACAGCTCC	ATAAACATAGCTAGTGTGGGTAC
ACGTCGATTGTCGAGG	TATTTGTATCGATCACACC
GCTAACAGCTCCATA	AACATAGCTAGTGTGGGTAC
ACGTCGATTGTCGAGGTAT	TTGTATCGATCACACC
GCTAACAGCTCCATAAAC	ATAGCTAGTGTGGGTAC
ACGTCGATTGTCGAGGTATTTG	TATCGATCACACC
GCTAACAGCTCCATAAACATA	GCTAGTGTGGGTAC
ACGTCGATTGTCGAGGTATTTGTAT	CGATCACACC
GCTAACAGCTCCATAAACATAGCT	AGTGTGGGTAC
ACGTCGATTGTCGAGGTATTTGTATCGA	TCACACC

Figure 11.

Lipman-Pearson alignment of RCNMV and TBSV coat protein sequences.

Lipman-Pearson Protein Alignment
Ktuple: 2; Gap Penalty: 4; Gap Length Penalty: 12

Seq1(1>389) tbsvtbs.PRO	Seq2(1>340) rcnmvdia.PRO	Similarity Index	Gap Number	Gap Length	Consensus Length
(64>387)	(8>338)	26.9	4	7	331

```
       ↓70         ↓80         ↓90         ↓100        ↓110        ↓120
KKQQMINHVGGTGGAIMAPVAVTRQLVGSKPKFTGRTSGSVTVTHREYLSQVNNSTGFQV
K.:Q. :. . T .: : .VA:. . .  . . . . : .: H : :  V .S. .:.
KSKQRSQPRNRTPNTSVKTVAIPFAKTQIIKTVNPPPKPARGILHTQLVMSVVGSVQMRT
  ↑10         ↑20         ↑30         ↑40         ↑50         ↑60
       ↓130        ↓140        ↓150        ↓160        ↓170        ↓180
NGGIVGNLLQLNPLNGTLFSWLPAIASNFDQYTFNSVVLHYVPLCSTTEVGRVAIYFDKD
N.G  .: ::LNP N :LF: L:  A:N:D Y :::.:L:YVPL :. : GRVA: .D D
NNGKSNQRFRLNPSNPALFPTLAYEAANYDMYRLKKLTLRYVPLVTVQNSGRVAMIWDPD
  ↑70         ↑80         ↑90         ↑100        ↑110        ↑120
       ↓190        ↓200        ↓210        ↓220        ↓230        ↓240
SEDPEPADRVELANYSVLKETAPWAEAMLRVPTDKIKRFCDDSSTSDHKLIDLGQLGIAT
S:D:.P..R E::.YS   .TA ...  L :P:D:  RF .D::T D:KL:D:GQL :.T
SQDSAPQSRQEISAYSRSVSTAVYEKCSLTIPADNQWRFVADNTTVDRKLVDFGQLLFVT
 ↑130        ↑140        ↑150        ↑160        ↑170        ↑180
       ↓250        ↓260        ↓270        ↓280        ↓290        ↓300
YGGAGTNAVGDIFISYSVTLYFPQPTNTLLSTRRLDLAGALVTASGPGYLLVSR---TAT
.:G::. ..GDIF:...V.:  PQPT.:::  .  :DL:G:L.: .GP:YL: :    T::
HSGSDGIETGDIFLDCEVEFKGPQPTASIVQKTVIDLGGTLTSFEGPSYLMPPDAFITSS
  ↑190        ↑200        ↑210        ↑220        ↑230        ↑240
          ↓310        ↓320        ↓330        ↓340        ↓350
VLTMTFRATGTFVISGTYRCLTATTLGLAG--GVNVNSITVVDNIG-TDSAFFINCTVSN
 :.:   .:GT:::: .  C T:.::.::G  .:  :: :. :: . ..S  F.:..V :
SFGLFVDVAGTYLLTLVVTCSTTGSVTVGGNSTLVGDGRAAYGSSNYIASIVFTSSGVLS
  ↑250        ↑260        ↑270        ↑280        ↑290        ↑300
  ↓360        ↓370        ↓380
LPSVVTFT-STGITSATVHCVRATRQNDVSL
 .: V F: S:G:.. ::  R .: N.  L
TTPSVQFSGSSGVSRVQMNICRCKQGNTFIL
  ↑310        ↑320        ↑330
```

Figure 12.

Beta plot - Chou-Fasman

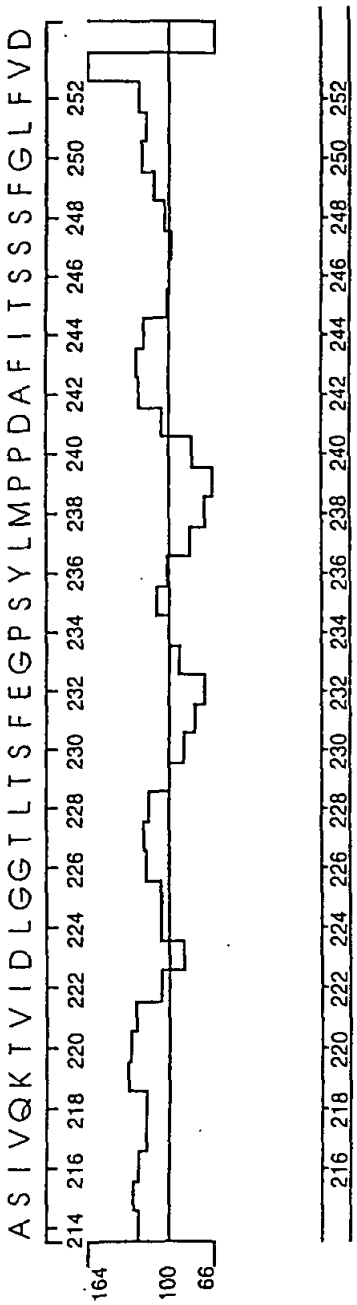

Figure 13.

16/17

```
                        220       230       240
          AA        |ASIVQKYVIDLGGTLTSFEGPSYLMPP
          PHD sec   | HHHHHEEEE   EEEE    EEEEE
          Rel sec   |145432244525515625586487624
detail :
          prH sec   |466655321111100000000000000
          prE sec   |101123456632246752212688753
          prL sec   |422221112246642237787311246
subset :  SUB sec   |..H.....E.LL.EE.LLLL.EEE..
```

Abbreviations :

AA : amino acid sequence
H : helix
E : extended (sheet)
blank : other (loop)
PHD : Profile network prediction HeiDelberg
Rel : Reliability index of prediction (0-9)
prH : probability for assigning helix
prE : probability for assigning strand
prL : probability for assigning loop
SUB : a subset of the prediction, for all residues with an average expected accuracy of >82%

Figure 14.

(a) Sequence of RCNMV Coat Protein Spanning The Potential Insertion Site With Introduced Base Changes and New Restriction Sites: (sequence starts at nt 3070)

```
S  I  V  Q  K  T  V  I  D  L  G  G  T  L  T  S  F
AGCATCGTACAGAAAACTGTAATTGATCTCGGTGGGACACTCACTTCTTTC
      ↓  ↓                          ↓  ↓
     GTGCAC                        GTTAAC
     ApaLI                         HpaI
```

(b) Series of Sequences to be Inserted Between the Restriction Sites to Insert the MUC1(16) Epitope at Various Locations

```
G  V  T  S  A  P  D  T  R  P  A  P  G  S  T  A
GGTGTTACTTCTGCTCCTGATACTAGACCTGCTCCTGGTTCTACTGCT
CCACAATGAAGACGACCACTATGATCTGGACGAGGACCAAGATGACGA
```

GAAAACTGTA	ATTGATCTCGGTGGGACGTT
ACGTCTTTTGACAT	TAACTAGAGCCACCCTGCAA
GAAAACTGTAATT	GATCTCGGTGGGACGTT
ACGTCTTTTGACATTAA	CTAGAGCCACCCTGCAA
GAAAACTGTAATTGAT	CTCGGTGGGACGTT
ACGTCTTTTGACATTAACTA	GAGCCACCCTGCAA
GAAAACTGTAATTGATCTC	GGTGGGACGTT
ACGTCTTTTGACATTAACTAGAG	CCACCCTGCAA
GAAAACTGTAATTGATCTCGGT	GGGACGTT
ACGTCTTTTGACATTAACTAGAGCCA	CCCTGCAA
GAAAACTGTAATTGATCTCGGTGGG	ACGTT
ACGTCTTTTGACATTAACTAGAGCCACCC	TGCAA

PCT / GB00 / 03500

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WC1A 2RA

11/09/00